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METHODS OF DETERMINING BOUND WATER IN PLANT TISSUE [1]

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INTRODUCTION

As a part of the research on the physiology of corn which was started when the European corn borer invaded Ohio, a study was made of the water content of different varieties of corn. This study included an attempt to find out the different ways in which water was held by the corn plant. In these studies, measurements were made of the amount of water removed from the tissues by pressure and the amount of bound water in the expressed sap. These measurements were suggested by the work of Newton and Gortner (*17*)[3] and Newton (*16*) on winter hardiness in wheat.

Unsatisfactory results were obtained in the measurements of the bound-water content of expressed sap from corn tissue by the cryoscopic method used by Newton and Gortner (*17*). Some determinations indicated no bound water in the sap; others showed a small percentage of bound water but satisfactory checks could not be obtained. These results were contrary to expectation, since the sap from leaf tissue was found to contain 4 to 5 per cent of colloidal material, largely proteins.

Before continuing work on the water content of corn, it was necessary, therefore, to test this method further, to try out other methods that had been suggested, and possibly to devise new ones; in other words, to make a critical study of methods used in determining bound water. Only those methods that appeared to be best suited for measuring bound water in plant tissues and in plant saps were considered.

DEFINITION OF BOUND WATER

A satisfactory definition of bound water can hardly be made. All water that is not free water, that is, that does not show some of the common properties of liquid water, may be considered as bound water. Foote and Saxton (*5*, *6*) recognize free, capillary or adsorbed, and combined water in such substances as lampblack, silica, alumina, and ferric oxide when mixed with water. They base their conclusions on the results of dilatometer measurements of the amount of water that will change to ice. Bouyoucos (*1*), also using dilatometer measurements, classified the water in certain soils as free and unfree. He states that unfree water may be either capillary adsorbed or combined.

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[2] The writer takes pleasure in acknowledging the helpful suggestions of F. D. Richey, of the Division of Cereal Crops and Diseases, during the preparation of this paper.

[3] Reference is made by number (italic) to Literature Cited, p. 686.

Newton and Gortner (*17*) divide the water in plant saps into free water and bound water. Their method of determination is based upon the assumption that bound water will not dissolve sucrose.

In plant tissues and in plant saps where there is material in true solution, such as sugars and salts, water may be held by hydration of molecules and ions and by osmotic phenomena. Maximow (*12*) and Rosa (*21*) discuss these forms of water. Gortner (*7*) considers water of imbibition and water that will not dissolve sucrose as bound water. Meyer (*13*) considers water that can not be removed by pressure from the tissue after certain treatments as bound water. From these examples and from many others that might be cited it seems that no single standard has been established and that water may be considered as bound when it is held or contained in the material in any one of several different ways.

EVIDENCE FOR EXISTENCE OF BOUND WATER

There is considerable evidence in physical chemistry for the existence of bound water. The hydration of sucrose or the association of a certain number of water molecules with each sugar molecule is an example. Philip (*19*) states that 5 molecules of water are associated with each molecule of sugar, and Findlay (*4*) and Scatchard (*22*) consider 6 as the correct number. The hydration of sucrose can be proved by several separate and distinct methods by which actual measurements can be made. All methods indicate that the number of associated water molecules is either 5 or 6. Ions also may be hydrated. Water held by ions or molecules does not have some of the properties ordinarily shown by liquid water, such as vapor pressure, osmotic phenomena, etc., and may be considered as bound water.

Many substances in a state of fine subdivision, such as carbon black, silica gel, platinum black, and alumina, have the property of adsorbing substances, including water. The adsorbed water may be in a thin layer around the particles or in the minute spaces between the particles. It is held by very strong forces and may have properties quite different from those of liquid water. Those substances in plant tissues and in plant saps which are in a similar state of subdivision may also adsorb water. Adsorbed water may be considered as bound water.

In sols like gum arabic, which do not set to a rigid gel, some of the water is associated with the particles, probably similarly to water of hydration or adsorbed water. Water so held and also that in rigid gels like agar, gelatin, and silica gel and in other similar plant or animal products is considered by Gortner (*7*) as bound water. Water of imbibition held by substances like Laminaria, fibrin, starch grains, and mucilages is also considered by him as bound water. Changes in water content in these substances usually are associated with viscosity changes. The force required to remove water held by these substances is very great. Meyer (*13*) measured the water forced out of plant tissues by pressure after freezing at $-15°$ to $-20°$ C. and at $-57°$. He considered that the water forced out without freezing corresponds roughly to the free water, and that that forced out after freezing, together with that left in the tissue, is bound in varying degrees.

Bound water does not exist in definite proportions relative to the solid material of the system, but as a ratio between bound water and free water. The ratio may be changed quickly by varying the temperature, acidity, surface energy, presence of electrolytes, pressure, etc. Various methods of measuring bound water have been suggested.

This paper reports the results of an attempt to simplify and standardize several of those that appear to be best suited for measuring bound water in plant tissues and in plant saps, as a preliminary to the determination of bound water in corn tissues.

METHODS OF MEASURING BOUND WATER

In most of the methods so far used, total water and free water are measured and the bound water is found by difference, the bound water being the water held by the solid part of the material in one or more of the several different ways discussed above.

THE CRYOSCOPIC METHOD

The cryoscopic method was introduced by Newton and Gortner (*17*). The theory involved is the assumption that bound water does not dissolve sucrose.

Apparatus and Technic

A freezing-point apparatus, a refractometer, and some means of obtaining suitable samples of expressed sap are necessary in making determinations on plant material by this method. The writer modified the procedure followed by Newton and Gortner (*17*) and thereby shortened the laboratory manipulation but increased the amount of calculating necessary. In this modified method accurate weighings of the liquid and of the sucrose are both eliminated. The amount of sucrose added to the liquid, on which the excess depression of the freezing point depends, is determined with the refractometer used to obtain the total solid content of the material, as described by Gortner and Hoffman (*8*).

The modified procedure substitutes one reading with the refractometer for two analytical weighings. The method is as follows: Determine the refractive index and the depression of the freezing point of the original liquid; add sucrose to a similar portion, about 3 g to 10 ml; and after the sucrose is dissolved determine the refractive index and freezing-point depression of this mixture. Several readings with the refractometer are made on each sample and the results averaged, and duplicate freezing-point measurements are made on separate portions of each mixture. The refractometer readings are corrected to a temperature of 20° C. and also for any difference between actual total solid content determined by vacuum oven drying and that indicated by the reading with the refractometer. From these data the amount of bound water is calculated by a formula modified slightly from the one given by Newton and Gortner (*17*).

Determining Amount of Sucrose Added

The refractometer was first used by Gortner and Hoffman (*8*) to determine the total solids in plant saps. This determination is based on the assumption that total solids in the sap have a refractive index very similar to that of sucrose. This assumption is true of rather dilute sols or solutions of dextrin, starch paste, glucose, fructose, gum arabic, and similar compounds, and of many plant saps. Before many determinations on any one substance are made, however, checks should be provided by vacuum oven drying. In some plant saps refractometer determinations are too high. Sap from blade tissue of corn gives values from 6 to 14 per cent too high, depending on the

season of the year, while readings on sap from stem tissues of the same plants agreed almost exactly with the results of vacuum oven drying.

The amount of sucrose added to a plant sap or to a gum arabic sol can be determined by the increase in refractive index, if the grams of total solids per 1,000 g of solvent are used instead of the percentages of total solids; that is, the grams of total solids per 1,000 g of solvent in the original sap subtracted from the grams of total solids per 1,000 g of solvent after sucrose was added will give the grams of sucrose per 1,000 g of solvent which was added. This method is the most convenient way of expressing the concentration of sucrose solutions when their freezing point is considered. From this value the additional depression of the freezing point due to the sucrose is determined and the percentage of bound water in the material calculated.

The refractive index, the percentage of total solids, and the grams of total solids per 1,000 g of solvent are given in Table 1. These data are based on the refractive indices of sucrose solutions given by Browne (*2*). The grams of total solids per 1,000 g of solvent were calculated by simple proportion from the percentages of sucrose and the percentages of water. Temperature corrections added to all values when readings are made above 20° C. are given in Table 2. These data should be put in graphic form to be useful in determining the concentration of the solutions or mixtures.

TABLE 1.—*Percentage by weight, refractive index, and grams of total solids per 1,000 g of solvent of sucrose solutions*

Percentage by weight	Refractive index at 20° C.[a]	Grams per 1,000 g solvent	Percentage by weight	Refractive index at 20° C.	Grams per 1,000 g solvent	Percentage by weight	Refractive index at 20° C.	Grams per 1,000 solvent
1	1.3344	10.1	15	1.3557	176.5	28	1.3775	388.9
2	1.3359	20.4	16	1.3573	190.5	29	1.3793	408.5
3	1.3374	30.9	17	1.3590	204.8	30	1.3811	428.6
4	1.3388	41.7	18	1.3606	219.5	31	1.3829	449.3
5	1.3403	52.5	19	1.3622	234.6	32	1.3847	470.6
6	1.3418	63.8	20	1.3639	250.0	33	1.3865	492.5
7	1.3433	75.3	21	1.3655	265.8	34	1.3883	515.2
8	1.3448	87.0	22	1.3672	282.1	35	1.3902	538.5
9	1.3464	98.9	23	1.3689	298.7	36	1.3920	562.5
10	1.3479	111.1	24	1.3706	315.8	37	1.3939	587.3
11	1.3494	123.6	25	1.3723	333.3	38	1.3958	612.9
12	1.3510	136.4	26	1.3740	351.4	39	1.3978	639.3
13	1.3526	149.4	27	1.3758	369.9	40	1.3997	666.7
14	1.3541	162.8						

[a] From Schönrock's table as given by Browne (*2*, *p.* 64).

TABLE 2.—*Temperature correction for total solids by the refractometer*

[Add the value to the total solids to convert values to 20° C. Computed from Stanek's correction table as given by Browne (*2*)]

Temperature (°C.)	Correction for indicated number of grams of total solids per 1,000 g of solvent							
	52.6	111.1	176.5	250.0	333.3	428.6	538.5	666.7
21	0.6	0.8	0.8	0.9	0.9	1.0	1.1	1.2
22	1.3	1.6	1.6	1.8	1.9	2.0	2.2	2.3
23	1.9	2.2	2.4	2.6	2.8	3.0	3.2	3.5
24	2.5	2.9	3.1	3.4	3.6	4.0	4.3	4.7
25	3.2	3.6	3.8	4.3	4.7	5.1	5.5	6.0
26	3.8	4.3	4.6	5.1	5.6	6.1	6.6	7.2
27	4.5	5.1	5.4	6.0	6.5	7.1	7.8	8.5
28	5.3	5.9	6.2	6.9	7.6	8.3	9.0	9.8
29	6.0	6.7	7.2	7.8	8.5	9.4	10.2	11.1
30	6.7	7.4	8.2	8.9	9.7	10.5	11.5	12.5

A comparison was made between this method of determining the amount of sucrose added to gum arabic solutions and the method of actually weighing the solution and the sucrose. The concentration of the gum arabic solution was determined with the refractometer and enough solution to contain 10 g of water was weighed out in a small weighing bottle. To this was added a weighed amount of sucrose. After the sucrose was dissolved the refractive index of the sugar-gum arabic mixture was determined. The amount of sucrose added was found from the two refractometer readings. The comparative data from a number of determinations which are given in Table 3 show that this method is essentially as accurate as weighing the sugar.

TABLE 3.—*Comparison of weighing and the refractive index method of obtaining the concentration of sucrose added to a gum arabic solution*

Gum arabic sol, percentage of total solids	Gum arabic	Gum arabic plus sucrose	Sucrose by difference	Actual amount of sucrose added
Per cent	*G per 1,000*	*G per 1,000*	*G per 1,000*	*G per 1,000*
9.6	105.5	449.4	342.9	342.2
9.6	105.5	449.4	342.9	342.2
9.6	105.5	448.4	341.9	342.2
9.6	105.5	449.5	342.1	342.2
9.6	105.5	452.0	344.6	342.2
9.6	105.5	449.0	341.6	342.2
Mean			342.6	342.2
9.8	107.7	210.2	102.5	100.0
9.8	107.7	308.0	200.3	200.0
9.8	107.7	410.9	303.2	300.0

If the refractometer does not give the true measure of total solids in the material, the calculation of the results is somewhat more complicated. The following data, taken from determinations on sap from blade tissue of corn, illustrate how the calculations are made. Refractometer readings and the determinations of total solids by vacuum oven drying were made on the original sap, and refractometer readings on the sap after the sugar was dissolved. Exactly 3 g of sucrose was added to 10 ml of sap. The results further corroborate the fact that the amount of sucrose added can be found from refractometer readings on the sap.

COMPARISON OF REFRACTIVE INDEX METHOD AND WEIGHING FOR DETERMINING THE AMOUNT OF SUCROSE ADDED TO PLANT SAP

Refractive Index Method

Refractometer reading on original sap = 1.3604 at 20° C.
Percentage of total solids by refractometer = 17.7.
Percentage of total solids by vacuum oven = 15.1.
Refractometer reading corresponding to 15.1 per cent total solids = 1.3559 at 20° C.
Correction to apply to refractometer reading 1.3559 − 1.3604 = −0.0045.
Refractometer reading after adding sucrose = 1.3924 at 20° C. (3 g to 10 ml of sap).
Corrected refractometer reading after adding sugar = 1.3879 at 20° C.
Grams per 1,000 g solvent corresponding to π = 1.3879 = 510
Grams per 1,000 g solvent corresponding to π = 1.3559 = 178

Grams sucrose per 1,000 g solvent added = 332

Weighing method

3 g sucrose to 10 ml original sap.
Specific gravity of original sap=1.070 at 20° C.
3 g sucrose to 10.70 g sap.
10.70 g sap contain (1.00–0.151) × 10.70, or 9.084 g water.
3 g sucrose to 9.084 g water, or 330 g sucrose to 1,000 g water.
Grams sucrose per 1,000 g solvent added=330.

Determining Depression of Freezing Point of Sucrose Solutions

The additional depression of the freezing point of the mixture, due to the added sucrose, is proportional to the amount of sucrose added. Newton and Gortner (*17*) used 2.085° C. as the molecular depression for sucrose, assuming 6 molecules of water of hydration for each molecule of sucrose. (Data of Scatchard (*22*).) A number of determinations were made to check this value, but the observed depressions were less than the calculated.

Table 4 gives the results of a series of these determinations. Each of the observed depressions is the average of four separate determinations on each concentration of the sucrose solution. The values calculated on the assumption that a molecular solution freezes at −2.085° C. are given in one column. If both the observed and the calculated values are plotted, two parallel lines are formed, that for the observed depressions being about 0.037° C. lower than the other. This difference may be due to some systematic error in all the freezing-point measurements, such as the purity of the sucrose used, the degree of undercooling, or the calibration of the thermometer. The values are very consistent, however, and the same procedure was used in making all the freezing-point measurements. These values accordingly have been used in this work rather than those given by the other authors.

Table 4.—*Comparison of actual determinations of the freezing point of sucrose solutions and calculated values of the same solution*

Percentage of sugar by weight	Sucrose per 1,000 g of water	Observed depression of freezing point	Calculated depression of freezing point	Difference
Per cent	*Grams*	*° C.*	*° C.*	*° C.*
14	162.8	0.955	0.992	0.037
16	190.5	1.124	1.161	.037
18	219.5	1.292	1.337	.045
20	250.0	1.485	1.523	.038
22	282.1	1.685	1.719	.034
25.5	342.2	2.054	2.085	.031

The percentages of free water in different concentrations of sucrose solutions are given in the following tabulation:

200 g of sucrose per 1,000 g solvent..... 94.5 per cent of free water.
250 g of sucrose per 1,000 g solvent..... 93.1 per cent of free water.
300 g of sucrose per 1,000 g solvent..... 91.7 per cent of free water.
350 g of sucrose per 1,000 g solvent..... 90.3 per cent of free water.
400 g of sucrose per 1,000 g solvent..... 90.0 per cent of free water

These values were calculated from the molecular weights of water and of sucrose and the actual freezing points of the various sucrose solutions as given in Table 4.

The corrections for undercooling in determining freezing points, taken from Harris (*9*), are included here (Table 5) so that complete data will be available for making determinations of bound water by the cryoscopic method.

TABLE 5.—*Undercooling correction factor for freezing-point determinations*

Tenth degrees	Whole degrees			Tenth degrees	Whole degrees		
	0	1	2		0	1	2
0.0	0.000	0.987	0.975	0.5	0.993	0.981	0.968
.1	.998	.986	.973	.6	.992	.980	.967
.2	.997	.985	.972	.7	.991	.978	.966
.3	.996	.983	.971	.8	.990	.977	.965
.4	.995	.982	.970	.9	.988	.976	.963

CALCULATING RESULTS

The percentage of bound water in the liquid or solution is calculated by a formula similar to the one given by Newton and Gortner (*17*).

$$\frac{\Delta_2-(\Delta_1+K_m)}{\Delta_2-\Delta_1}\times C = \text{ per cent bound water}$$

Δ_2 is the depression of the freezing point after adding sucrose.

Δ_1 is the depression of the freezing point of the original solution.

K_m is obtained from the difference between the two concentrations, corresponding to the two refractive index readings (Table 1), and the depression of the freezing point of a sucrose solution (Table 4.)

C is the percentage of free water in a sucrose solution of the concentration used. (Table 5.)

Inasmuch as exactly 342.2 g of sucrose is not always used, K_m will not always be 2.085° C., but some other value, depending on the amount of sucrose added. C also will vary with the amount of sucrose added. These values can best be obtained from graphs of the values for various strengths of sucrose approximating molecular concentrations, given in Tables 1 and 4 and in the tabulation on p. 674.

ADVANTAGES AND DISADVANTAGES OF THE METHOD

The advantage of the modified cryoscopic method is that the actual laboratory procedure is shortened, although the calculations of the results are more complicated. When only a few determinations are to be made, the original method is superior, but when many measurements are to be made, graphs of the various constants used in the determination can be used as an aid in calculation, and the shorter laboratory procedure is the more efficient. From six to eight different saps or solutions can be analyzed with duplicate freezing-point depression determinations in a half day with this short method.

The disadvantage of the cryoscopic method is that it can be used only with liquid or semiliquid material. This is particularly a disadvantage when plant tissues are being studied, as the amount of bound water in the tissue is the most important consideration. Furthermore, the method measures only water that is not free to dissolve sucrose. If water held by gelatin and agar is considered as

bound water, then this method fails to give a real measure of bound water, since much of the water held by those substances will dissolve sucrose. Gortner (7) has outlined certain other objections to the method. It probably gives minimum values for bound water. It presupposes, (1) that bound water is not changed to free water by the freezing process, (2) that the addition of sucrose does not alter the bound-free water ratio, (3) that none of the water that is firmly associated with the colloids is free to dissolve sucrose, (4) that there is no hydrolysis of the sucrose by acids or by enzymes in the material, and (5) that no adsorption of sucrose by the solid matter occurs. None of these assumptions is probably entirely correct.

THE CALORIMETER METHOD

The calorimeter method was first introduced by Müller-Thurgau (*14*, *15*) and recently has been used by Thoenes (*23*) and Robinson (*20*). The method is based on the assumption that bound water does not freeze. Since free water changes to ice and it requires 79.75 calories to melt each gram of ice while unfrozen water below 0° C. has a specific heat of approximately 1.00, the amount of heat required to melt the mass of frozen material can be used to determine how much water was frozen.

Apparatus and Technic

The equipment necessary for determinations by this method consists of a calorimeter thermometer, a calorimeter, freezing tubes for the tissue or material, low-temperature thermometers, and a freezing cabinet or refrigerator where a temperature as low as −25° C. or lower can be maintained. Quart-size all-steel thermos food jars make excellent calorimeters for plant-tissue work.

In making determinations the material is weighed and placed in freezing tubes. The tubes are about 4 inches long, open at both ends, and about one-half inch larger in diameter at one end than at the other. This kind of tube is necessary in order to make a quick transfer of the frozen material to the calorimeter without thawing any of it, as is likely to occur if the tubes are of uniform diameter. Both ends are closed tightly with rubber stoppers. The tubes of material are placed in a freezing room or cabinet at about −25° C. overnight. Before the measurements are made the tubes are taken in the hand and the frozen material is thawed around the tube and pushed to the large end, where it is not in contact with the wall of the tube. One tube has a thermometer in it. When the tubes have come to equilibrium again, as indicated by the thermometer, they are taken one at a time and the contents quickly transferred to the water in the calorimeter. The volume of water and its temperature to 0.01° C. are known. The tissue and water are stirred until equilibrium is reached and the fall in temperature of the mixture is determined.

Any loss of heat from the calorimeter during the stirring is reduced to a minimum by adjusting the temperature of the water in the calorimeter so that the fall in temperature will be about equal on either side of room temperature. This range can be found out by a preliminary determination on similar material. The material is stirred steadily until the fall in temperature in the bottle ceases. Finely minced or ground material comes to equilibrium much more quickly than large chunks. The time required to reach equilibrium is only a few minutes. Six to eight determinations can be made in an hour.

Calibrating the Calorimeter System

Although the actual determinations are quite simple, the work necessary to calibrate the system and to calculate the results is considerable. The factor for the calorimeter system is obtained by putting ice into water in the calorimeter and determining how much heat is necessary to melt it and raise the temperature of the resulting water to the equilibrium temperature of the system. For a known weight of ice this amount of heat can be calculated from the latent heat of fusion of ice, 79.75 calories, and the specific heat of water. Any difference between the observed and the calculated values must be due to transfer of heat from the calorimeter system. The quotient of these two values gives a factor for converting the observed values to the true values for the material in the experiments.

A 25 g sample of the material has been used. By putting about 25 g of water in the freezing tubes and carrying out the determinations just as was done with tissue or other material, a factor for the calorimeter system was determined which included any error due to taking up heat while transferring the material from the freezing chamber. Table 6 gives the observed and calculated values of heat necessary to melt the ice and warm the water to the equilibrium of the system, and the corresponding factor for the system. This factor applies, of course, only to the system of thermos bottle, stirrer, thermometer, etc., used in these particular experiments.

Table 6.—*Determination of the factor for the calorimeter system*

Ice used (grams)	Calories required to melt ice and warm water to equilibrium	Calories given up by 500 cc water cooling to equilibrium	Factor for the calorimeter system	Ice used (grams)	Calories required to melt ice and warm water to equilibrium	Calories given up by 500 cc water cooling to equilibrium	Factor for the calorimeter system
23.96	2,768.4	2,587.4	1.0700	24.05	2,774.8	2,602.4	1.0662
21.40	2,481.7	2,317.7	1.0708	22.59	2,613.5	2,442.6	1.0700
24.11	2,779.6	2,597.4	1.0701	24.22	2,793.3	2,617.4	1.0672
23.58	2,719.6	2,542.5	1.0697	24.10	2,778.9	2,607.4	1.0658
23.73	2,736.1	2,557.4	1.0699				
24.20	2,787.1	2,612.4	1.0669	Mean			1.0684
23.50	2,710.4	2,542.5	1.0660				

Calculating Results

The fall in temperature of the water in the calorimeter multiplied by the specific heat of water multiplied by the volume of water and the factor for the calorimeter system gives the total number of calories required to melt the ice and warm the water and dry matter of the tissue up to the equilibrium temperature. This heat is used in several ways: (1) To warm the dry matter of the tissue from its temperature when placed in the calorimeter to the equilibriun temperature. This amount can be determined from the specific heat of the material and the change in temperature. (2) To warm the water in the tissue from the freezing (thawing) temperature of the tissue to the equilibrium temperature. This amount can be determined from the amount of water, its specific heat,and the change in temperature. The approximate freezing point of similar tissue should be known. The determinations from (1) and (2) are added, and this sum is subtracted from

the total number of calories required. The remainder (3) is the number of calories required (*a*) to warm the unfrozen water from its temperature when placed in the calorimeter to the freezing (thawing) point of the tissue, (*b*) to warm the ice from its temperature when placed in the calorimeter to the freezing (thawing) point of the tissue, and (*c*) to melt the ice.

The total water in the sample, the latent heat of fusion of ice (79.75 calories), and the specific heat of water and of ice are known. The amount of free or frozen water accordingly can be calculated by simultaneous equations. Letting X = free or frozen water, Y = bound or unfrozen water, and W = total water in the sample, then

$$X+Y=W \tag{1}$$

Let C be the number of calories required to warm the ice and the unfrozen or bound water to the melting (thawing) point and to melt the ice. T_o is the temperature of the material at the time it was placed in the calorimeter, and T_Δ is the freezing point of similar material. Let SI be the mean specific heat of ice, and let SBW be the mean specific heat of unfrozen or bound water. Then

$$C=X\,[79.75+SI\,(T_o-T_\Delta)]+Y\,[SBW(T_o-T_\Delta)] \tag{2}$$

Combining equations (1) and (2) and equating to X,

$$X=\frac{\dfrac{C}{SBW\,(T_o-T_\Delta)}-W}{\dfrac{79.75+SI(T_o-T_\Delta)}{SBW\,(T_o-T_\Delta)}-1}$$

or simplifying,

$$X=\frac{C-W[SBW\,(T_o-T_\Delta)]}{79.75-[T_o-T_\Delta\,(SBW-SI)]} \tag{3}$$

Now,

$$C=FNS\,(T-T_e)-[SW(T_\Delta+T_e)+sM(T_o+T_e)] \tag{4}$$

where F is the factor for the calorimeter system, N is the amount of water used in it (500 ml in this work), S is the mean specific heat of water for the temperature range indicated, T is the temperature of the water in the calorimeter before the sample was placed in it, T_e is the temperature in the calorimeter at equilibrium after the sample was introduced, s is the mean specific heat of the dry matter of the sample for the temperature range indicated, M is the amount of dry matter in the sample.

By combining equations (3) and (4) the complete formula becomes:

$$X=\frac{FNS\,(T-T_e)-[SW(T_o+T_e)+sM(T_o+T_e)]}{79.75-[T_o-T_\Delta(SBW-SI)]}$$

Bound water $=W-X$.

The individual calculations can be shortened somewhat by solving the denominator of the fraction for all values of T_o-T_Δ used in the

investigation, since this does not depend on the size of the sample or its water content. $SW(T_o+T_e)+sM\,(T_o+T_e)$ can also be shortened to $SM\,(T_o+T_e)$. S now is the mean specific heat of the sample and M is its green weight. The specific heat of the green sample can not be determined for the entire temperature range, T_o+T_e, since ice will form below the freezing point of the material. It can be determined with sufficient accuracy for most work by using the temperature range 0° C. to T_e and assuming that it does not change materially below 0° C., or it can be calculated from the specific heat of the dry matter of the sample.

Thoenes (*23*) and Robinson (*20*) have simplified the formula still further by assuming that the specific heat of ice is 0.500 and of water below zero 1.000. The formula that they use is given by Gortner (*7*).

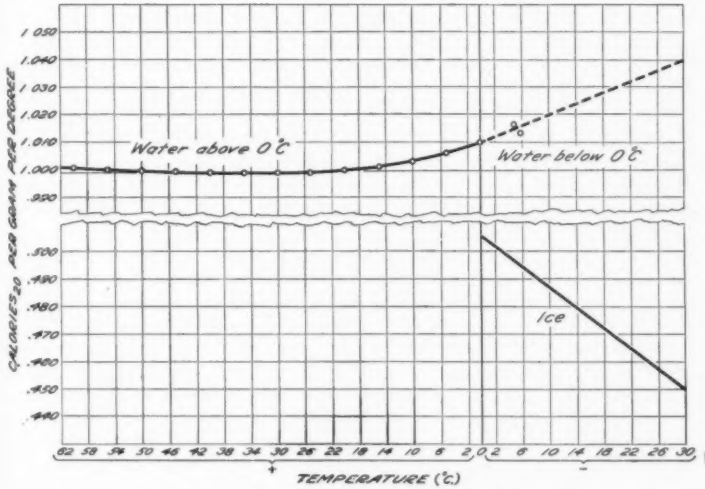

FIGURE 1.—Specific heat of water and of ice

Measurements by the Bureau of Standards (*3*) of the specific heat of ice, however, show that the specific heat of ice is decreasingly lower than 0.500 at temperatures much below 0° C. The specific heat of water below zero, on the other hand, increases as the temperature is lowered.

Table 7 gives the specific heat of water both above and below 0° C. and the specific heat of ice. The values for ice were taken from Dickinson and Osborne (*3*). The values for water above zero were taken from the Handbook of Chemistry and Physics (*10*). They were reduced to values at 20° C., to be comparable with ice. The values for water below zero were obtained by extending the curve of values from 0° to −30° as a straight line, assuming that the specific heat of water continues as a linear function of the temperature, as shown in Figure 1, no extensive data being obtainable.

TABLE 7.—*Specific heat of water and of ice*

[Calories$_{20}$ per gram per degree]

Temperature (° C.)	Water below zero	Ice	Water above zero	Temperature (° C.)	Water below zero	Ice	Water above zero
0	1.010	0.506	1.010	16	1.026	0.476	1.001
1	1.011	.504	1.009	17	1.027	.474	1.001
2	1.012	.502	1.008	18	1.028	.472	1.000
3	1.013	.500	1.008	19	1.029	.470	1.000
4	1.014	.498	1.007	20	1.030	.468	1.000
5	1.015	.496	1.006	21	1.031	.467	1.000
6	1.016	.495	1.005	22	1.032	.465	1.000
7	1.017	.493	1.005	23	1.033	.463	.999
8	1.018	.491	1.004	24	1.034	.461	.999
9	1.019	.489	1.004	25	1.035	.459	.999
10	1.020	.487	1.003	26	1.036	.457	.999
11	1.021	.485	1.003	27	1.037	.455	.999
12	1.022	.483	1.002	28	1.038	.454	.999
13	1.023	.481	1.002	29	1.039	.452	.999
14	1.024	.480	1.002	30	1.040	.450	.999
15	1.025	.478	1.001				

ADVANTAGES AND DISADVANTAGES OF THE METHOD

The calorimeter method of determining bound water has been very satisfactory. It has certain advantages in ease of technic, and it can be used for any kind of material, whether liquid, semiliquid, or solid. For these reasons it appears to be the best method so far suggested for measuring bound water in plant tissues. It measures water that does not freeze at the temperature to which it is exposed.

The calorimeter method is open to only one of the several objections that were listed under the disadvantages of the cryoscopic method, namely, the assumption that the bound water-free water ratio may not shift during the freezing process. In substances with no material in true solution most of the water is frozen at a few degrees below zero and a lower temperature does not crystallize any more of it. But where there is considerable material in true solution, as in plant tissues, more ice may separate as the temperature is lowered. For this reason all reported measurements of bound water in plant tissues or plant saps by this method should include a statement of the temperature at which the material was frozen, and comparisons should be made only between materials frozen at the same temperature.

THE DILATOMETER METHOD

The dilatometer method has been used to determine the amount of water that will freeze in certain systems at certain temperatures. Foote and Saxton (*5*, *6*) used this method in determining water held in lampblack, silica, ferric oxide, and alumina. Bouyoucos (*1*) determined free and unfree water in soils by this method. McCool and Miller (*11*) and Rosa (*21*) used it in determining the amount of water in plant tissues which would not freeze at certain temperatures. The principle involved in the method is the expansion that occurs when water changes to ice. This expansion is about one-tenth the volume of the water that changes to ice, and from it the amount of water that freezes can be determined. Bound water is found by difference from total water.

Apparatus and Technic

The determinations are carried out in flasks with long, narrow, graduated necks, called dilatometers. The dilatometers are immersed in freezing mixtures of any desired temperature. The material is covered with petroleum ether, which is immiscible with water. The expansion is obtained as freezing or cooling occurs by noting the change in the meniscus of the petroleum ether in the narrow neck.

No regular dilatometers were available, and cream-test bottles were used instead. They are suitable for most substances, but filling them with solid material is tedious. The material must be in small pieces to go through the narrow neck of the flask. They have one advantage over the form of dilatometer used by Bouyoucos (*1*), however, in that they are not hard to seal, having only one opening. The neck is graduated to 0.05 ml, and readings can be estimated to 0.01 ml. A 25+g sample of liquid material can be used in them very well.

The sample is introduced into the bottle, covered with petroleum ether, and the whole cooled in a freezing mixture to any desired temperature. The expansion due to ice formation gives the amount of water that freezes at this temperature. Bouyoucos (*1*) supercooled his soil samples to $-1.5°$ and $-4°$ C. and took the volume of the expansion at these temperatures. Rosa (*21*) made determinations at $-3°$, $-4°$, $-5°$, and $-6°$. Foote and Saxton (*5*, *6*) made readings on their dilatometers at a series of temperatures ranging from $0°$ to $-30°$.

Bouyoucos (*1*) considered the water in soils that froze at $-1.5°$ C. as free water, and that which froze at $-4°$ as capillary adsorbed water, and that unfrozen at $-4°$ as combined water. Foote and Saxton (*5*, *6*) could find no temperature where the different forms of water could be sharply separated. They expressed their results as curves and show that the amount of water that freezes increases as the temperature is lowered in some cases, while in others it is all frozen at a quite definite point. They further show that, after the water in some substances is frozen, the temperature may be raised to a point above which freezing occurred when cooling the material, without melting any of the ice. This means that ice may be superheated.

Procedure

The general procedure used is similar to that of other workers who have employed the dilatometer method, except that the order of taking the readings on the dilatometer was reversed. The material was placed in the dilatometer and frozen solid overnight, and the petroleum ether was then added. A series of readings on the volume of the material in the dilatometer as the temperature was changed was made, after which the material was allowed to melt and another series of readings was made on the volume as the temperature was changed. This procedure was necessary to prevent breaking the dilatometers, which were placed in a temperature cabinet and could not be shaken or stirred as freezing occurred. When the ether was added before freezing, ice formed first between the ether and the material, preventing expansion as freezing progressed, and the bottles were usually broken.

Calibrating the Dilatometer

Each dilatometer must be calibrated separately, since the glass in each is of different thickness and all do not have the same volume. The graduations on the neck are standard and uniform. The factor obtained represents the expansion due to ice formation and any other changes in volume of the glass vessel due to temperature changes, and thus the values are usually higher than if they were due to ice formation alone. Since the factor obtained from the calibration may vary with the rate and temperature at which the ice formed, it is essential that they be used at the same temperature, etc., as those for which they are calibrated.

In calibrating the dilatometers, 25 ml of water was placed in the dilatometer and frozen solid overnight in the freezing cabinet at −25° C. The dilatometers were filled with petroleum ether up to the top graduation on the neck and corked. After temperature equilibrium was reached the position of the meniscus in the graduated neck was recorded. The temperature of the cabinet was changed by about 5° intervals, and after temperature equilibrium had been reached, in two or three hours, the meniscus was again read. A series of such readings was obtained at intervals until the material was at a temperature at which it melted. After all the material had melted another series of readings was made at 0° and at intervals of about 5° up to room temperature. When the data were plotted two lines were obtained. One of these represented the change in volume of the unfrozen material and the other the change in volume of the frozen material, with changes in temperature. The distance between the two lines, which are usually straight and parallel, is the expansion in milliliters due to ice formation of the 25 ml of water. A graphic solution of the factor for one of the dilatometers is given in Figure 2.

In the determination of bound water in samples of various materials the procedure was the same as in determining the factor with water for the dilatometers. The change in volume then was corrected for the dilatometer used in accordance with the factor for it which had been determined. Thus it is essential that all determinations on bound water in different samples be carried out under the same conditions under which the dilatometers were calibrated.

Advantages and Disadvantages of the Method

This method can be used to measure bound water in almost any kind of material, either liquid or solid. With the cream-test bottles used as dilatometers, the method is practically restricted to liquid or semiliquid material, because of the small neck of the flask. The method is simple and accurate and involves no complicated calculation as do the other two methods, since results can be obtained by graphic solution. It is free from errors when properly used. The only precautions necessary are to see that the dilatometers are tightly stoppered to prevent evaporation of the petroleum ether, and to be certain that all the air is removed from the material before the readings are made. This latter is very difficult with plant tissue, especially mesophyll tissue containing intercellular spaces. One decided advantage of the method is that the amount of water that changes to ice at each temperature can be obtained. It measures water that will not freeze at different temperatures.

COMPARISON OF THE THREE METHODS OF MEASURING BOUND WATER

In order to compare these three methods, measurements of bound water by each method were made on exactly similar material. Gum arabic was chosen as material because it was the substance originally used by Newton and Gortner (*17*) and because a large supply could readily be obtained. All measurements in this comparison were made on the same lot of gum arabic, which was ground and thoroughly mixed to insure a uniform sample. It was originally intended to check all methods on several different materials, but this was found impossible because of lack of time. The results recently published by

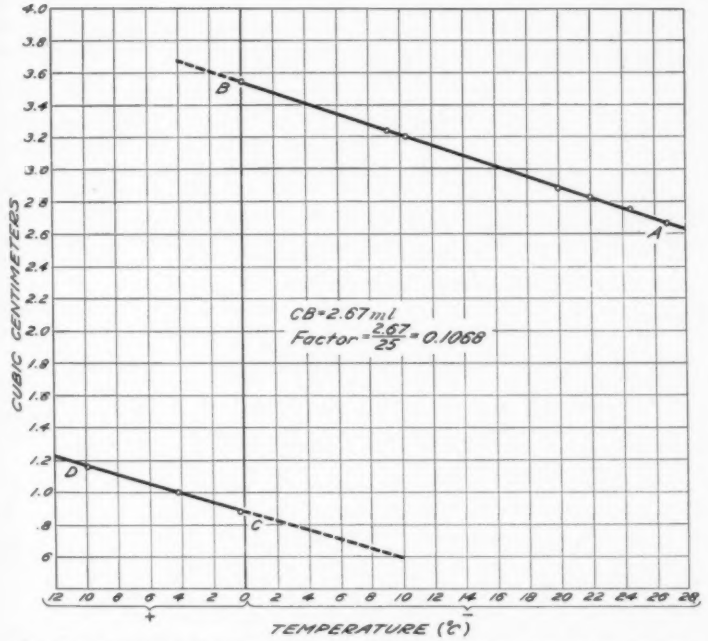

FIGURE 2.—Calibration of dilatometer No. 3: A–B=change in volume of 25 g of ice, petroleum ether, and the dilatometer with changes in temperature; C–D=change in volume of 25 ml of water, petroleum ether, and the dilatometer with changes in temperature; B–C=expansion due to ice formation

Newton and Martin (*18*) on the bound-water content of different substances by the cryoscopic method would suggest that some very interesting results might be obtained if the materials that they used were checked by several methods.

Experience soon showed that all measurements would have to be made as soon as possible after the solution was prepared and all on the same solution, as changes occur as the solutions stand and two solutions apparently made up in the same way may not have the same bound-water content. After a considerable number of preliminary experiments, a saturated solution (18.6 per cent) of gum arabic was prepared, and the values for bound water in Table 8 were all obtained on this solution.

TABLE 8.—*Comparison of the three methods of measuring bound water in an 18.6 per cent gum arabic solution*

CRYOSCOPIC METHOD

Sucrose added	K_m	C	Δ_2	Bound water
	° *C.*	*Per cent*		*Per cent*
260.8	1.552	92.9	1.827	10.84
403.9	2.431	88.9	3.018	15.59
305.5	1.830	91.6	2.182	12.23
267.2	1.592	92.6	1.922	13.00
289.0	1.728	92.0	2.035	11.10
309.2	1.852	91.5	2.173	10.92
				Mean = 12.28
				σ = 1.83
				PE_m = ±0.50

Δ_1=0.070° C.

CALORIMETER METHOD

T_o	T	T_s	$FNS(T-T_s)$	$SW(T_o+T^c)$	$sM(T_o+T_s)$	$79.75-T_o-T_\Delta(SBW-SI)$	Bound water
° *C.*	° *C.*	° *C.*	*Calories*	*Calories*	*Calories*	*Calories*	*Per cent*
−4.0	27.32	23.36	2113.32	578.87	74.33	77.7610	10.59
−3.8	27.92	23.98	2102.64	587.76	75.47	77.8630	11.97
−3.5	27.24	23.40	2049.28	569.14	73.08	78.0194	14.12
−3.4	27.54	23.60	2102.64	571.25	73.35	78.0703	11.07
−3.3	27.20	23.36	2049.28	564.06	72.43	78.1244	13.87
−3.2	28.54	24.70	2049.28	591.14	75.91	78.1752	15.80
						Mean = 12.90	
						σ = 2.02	
						PE_m = ±0.56	

F=1.0684; N=500 ml; s=0.566 cal.; W=21.00 g; m=4.80 g; T_Δ=0.10° C.

DILATOMETER METHOD

Dilatometer No.	Expansion 25 ml water on freezing	Factor	Expansion 25 ml gum arabic[a] on freezing	Bound water
	Ml		*Ml*	*Per cent*
1	2.47	0.0088	1.82	13.11
2	2.35	.0940	1.81	9.17
3	2.42	.0968	1.81	11.80
4	2.42	.0968	1.82	11.32
5	2.50	.1000	1.83	13.68
6	2.47	.0988	1.81	13.59
				Mean = 12.11
				σ = 1.74
				PE_m = ±0.48

[a] 25 ml gum arabic, sp. gr. 1.042, contained 21.20 g of water and 4.85 g of gum.

The mean values for bound water as determined by the three methods are in excellent agreement. The maximum difference is 0.79 ± 0.74 per cent of bound water for the calorimeter and dilatometer methods. This is only about 6.5 per cent of the total bound water as measured by the dilatometer method and is without statistical significance in view of its probable error. The variability, as measured by the standard deviation (σ), also was essentially the same for the three methods in these experiments. Too much reliability should not be placed on these values, however, as they were obtained from only six measurements. Refinements in the technic of the calorimeter method have been made since this comparison was finished. These have reduced the variation in repeated determinations

by that method. Thus, a series of determinations of bound water in samples of corn tissue had a standard deviation of only 0.81, or some 4.2 per cent of the indicated bound-water content.

An attempt was made to check the three methods on expressed sap from corn leaf tissue. The percentage of bound water by the calorimeter method was 14.8 per cent, by the dilatometer method 12.7 per cent, and by the cryoscopic method 5.8 per cent. All three methods were used on the same sample of expressed sap. The results by the calorimeter method and by the dilatometer method are in reasonably good agreement, but the results by the cryoscopic method are not. The cryoscopic method was very good for determining bound water in gum arabic solution. It has also been used with apparent success by other workers, Meyer (*13*) and Newton and Martin (*18*), on many different plant saps and other materials. However, as previously stated, it failed to give reliable results with expressed sap from corn tissue.

Since the cryoscopic method does not always give reliable results, determinations of bound water should be checked by different methods whenever possible.

If all three methods were equally accurate, the calorimeter method would apparently be the more useful, since it is easy to use with any type of material. The bound-water contents of a number of different substances determined by the calorimeter method are given in Table 9 as an illustration.

TABLE 9.—*Total water, free water, and bound water content of different materials, determined by the calorimeter method*

Material	Date	Total water	Free water	Bound water		Bound water per 100 g solid	Temperature at which material was frozen	Temperature at which material was determined	Treatment of tissue
	1930	*Per cent*	*Grams*	*Grams*	*Per cent*	*Grams*	*° C.*	*° C.*	
Buckeye twigs	Feb. 28	56.0	26.2	29.8	53.2	67.8	−23.0	−4.5	Shavings.
Buckeye buds	do	54.0	24.5	29.5	54.6	64.2	−23.0	−4.0	Whole.
Maple twigs	Mar. 4	44.0	20.4	23.6	53.6	42.2	−24.0	−5.5	Shavings.
Pine needles	Mar. 6	56.0	40.7	15.3	27.3	34.8	−23.0	−11.5	Ground.
Do	do	56.0	41.2	14.8	26.4	33.7	−23.0	−11.0	Minced.
Do	do	42.0	23.1	18.9	45.0	32.6	−23.0	−11.0	Press cake.
Do	Apr. 1	56.8	40.8	16.0	28.2	37.0	−22.9	−20.0	Minced.
Do	do	56.8	38.9	19.9	35.0	46.1	−12.0	−10.0	Do.
Carbon	Mar. 27	50.0	28.2	21.2	42.4	42.4	−23.0	−3.0	
Filter paper	do	73.0	61.6	11.4	15.6	42.5	−23.0	−4.2	
Starch paste	do	98.5	94.4	4.1	4.2	27.3	−23.0	−2.5	
Corn blade	Aug. 12	72.2	56.6	15.6	21.6	56.1	−25.0	−25.0	Do.
Do	do	72.2	56.8	15.4	21.3	55.4	−25.0	−25.0	Ground.
Corn sheath	do	78.3	67.9	10.4	13.3	47.9	−25.0	−25.0	Do.
Corn stem (upper)	do	78.3	69.5	8.8	11.2	40.5	−25.0	−25.0	Do.
Corn stem (middle)	do	80.0	73.8	6.2	7.8	31.0	−25.0	−25.0	Do.
Corn stem (lower)	do	80.9	74.5	6.4	7.9	33.5	−25.0	−25.0	Do.

To all three methods the objection may be made that they measure bound water at or near the freezing point. Since bound water is in equilibrium with the free water of the system, and this equilibrium may be changed by temperature, they do not give a true measure of the bound water present at ordinary temperatures. This objection is not serious when bound water is considered in relation to cold

resistance, since the bound-water content near freezing temperatures is the item wanted, but in studying drought resistance this objection might be a serious one.

SUMMARY

No satisfactory definition of bound water can be made. All water that is not free water, that is, that does not show some of the common properties of liquid water, may be considered as bound water. In the methods of determining bound water, free water is measured and bound water is found by difference. This paper presents the results of a study of three methods of measuring bound water in plant tissue, namely, the cryoscopic method, the calorimeter method, and the dilatometer method.

The theory involved in the cryoscopic method is the assumption that bound water is not free to dissolve sucrose. A determination of the increased depression of the freezing point of the material after sucrose is dissolved in it will indicate whether or not all the water present is free for the solution of sucrose. The chief disadvantage of the cryoscopic method is that it can be used only for liquid or semiliquid material. It is open to several other objections, the most important of which is that the addition of sucrose in molecular concentration to the material may change the bound-free water equilibrium or that the sucrose may be hydrolyzed or adsorbed by the material.

The theory involved in the calorimeter method is the assumption that bound water does not freeze. In this method the amount of water that changes to ice is determined by measuring the amount of heat necessary to thaw the frozen material. Owing to the great difference between the latent heat of fusion of ice and the specific heat of water, rather small quantities of ice can be measured. This method can be used on any kind of material, whether liquid, semiliquid, or solid.

The theory involved in the dilatometer method is also the assumption that bound water does not freeze. In this method the expansion of the material as freezing occurs is used to determine how much of the water changes to ice. This method can also be used on any kind of material, but great care is necessary to be sure that all air is removed from the material before it is frozen. Both the calorimeter and the dilatometer methods are open to the objection that the bound-free water equilibrium may be changed by the freezing of the material at the low temperature used.

The calorimeter method is recommended for the measurement of bound water in practically all materials, since it is easy, rapid, accurate, and reliable. Whenever possible, however, determinations should be made on the material by each of the several methods.

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ELSINOE ON APPLE AND PEAR[1]

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INTRODUCTION

This paper deals with the morphology and taxonomy as well as the history and distribution of *Plectodiscella piri* (*55*),[2] which causes an anthracnose of considerable importance, affecting apple (*Malus sylvestris* Mill.) and pear (*Pyrus communis* L.). Reference is made also to Sphaceloma, that is, the conidial stage of Plectodiscella, on other plants besides those named, most of which have not heretofore been reported as attacked by this group of fungi. Plectodiscella is shown to be a synonym of Elsinoe.

HISTORICAL REVIEW

The history of *Plectodiscella piri* as then known was reviewed by Jenkins and Horsfall (*31*) in 1929, and the Sphaceloma or conidial stage of the organism reported. This was recognized on the basis of conidia on leaves of apple and pear from Transcaucasia,[3] part of the collection on which the perfect stage of the fungus was discovered, and on conidia in a theretofore unidentified culture isolated by Osterwalder (*38*) from a fruit spot on Jonathan apple grown in Switzerland. He diagnosed the disease as Jonathan spot, which is ordinarily considered to be nonpathogenic. A publication of Zschokke (*57*) in which he mentioned the occurrence of the disease on Jonathan apple, as referred to by Osterwalder (*38*), was not available to Jenkins and Horsfall in 1929. Now at hand, however, it reveals that not only the Jonathan variety (pl. 1, A), but several others there illustrated show dark lesions which appear to be those of the Plectodiscella disease.

In the past two years the writer has diagnosed the anthracnose on fresh apples intercepted in transit[4] from Ireland (fig. 1), Italy, Switzerland, and Hungary, and also on apples imported from Portugal. In each case isolations of the pathogene have been made. The apples from Ireland and Italy have been referred to in earlier publications (*28*, *52*); those from Portugal, of the variety Reneta, were secured through the courtesy of Mathilde Bensaúde, of the Instituto Rocho Cabral, Lisbon. So far as the writer knows, this fungus has not been found in North America.

Although records are not available it is believed that *Plectodiscella piri* infects the twigs of apple and pear as well as the leaves and fruits. The larger lesions on the apple fruit shown in Figure 1 were brownish or

[1] Received for publication Dec. 8, 1931; issued May, 1932.

[2] Reference is made by number (italic) to Literature Cited, p. 697.

[3] Russia, Transcaucasia, near Sotschi, Aug. 28, 1913. N. N. Woronichin. Ex Herbarium, Institute of Mycology and Phytopathology, Leningrad, U. S. S. R. Contributed by N. N. Woronichin and A. Jaczewski.

[4] Intercepted by port inspectors of the Plant Quarantine and Control Administration, in passengers' baggage and in mail.

whitish at the center surrounded by carmine[5] or jasper red, while those on the other fruits examined were mostly madder brown or chestnut.

MORPHOLOGY

Plates 1, B, *a* and *b*, and 2, A, show *Plectodiscella piri* on apple and pear leaves from Transcaucasia,[6] on which conidia were found by Jenkins and Horsfall (*31*), and Plate 3, A and B, those of the fungus on pear leaves from Italy.[7] Although conidia were not abundant on the Transcaucasian material, they practically covered the acervuli examined on the Italian specimens. (Pl. 3, C and D.) In all cases the conidia seen have been of the various shapes, sizes, and colorations

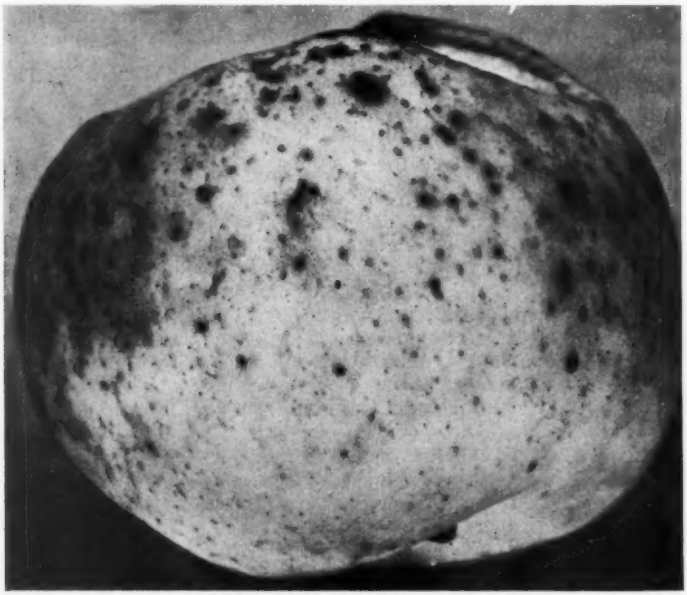


FIGURE 1.—Apple fruit from Ireland affected by anthracnose. Slightly enlarged. Photograph by J. F. Brewer

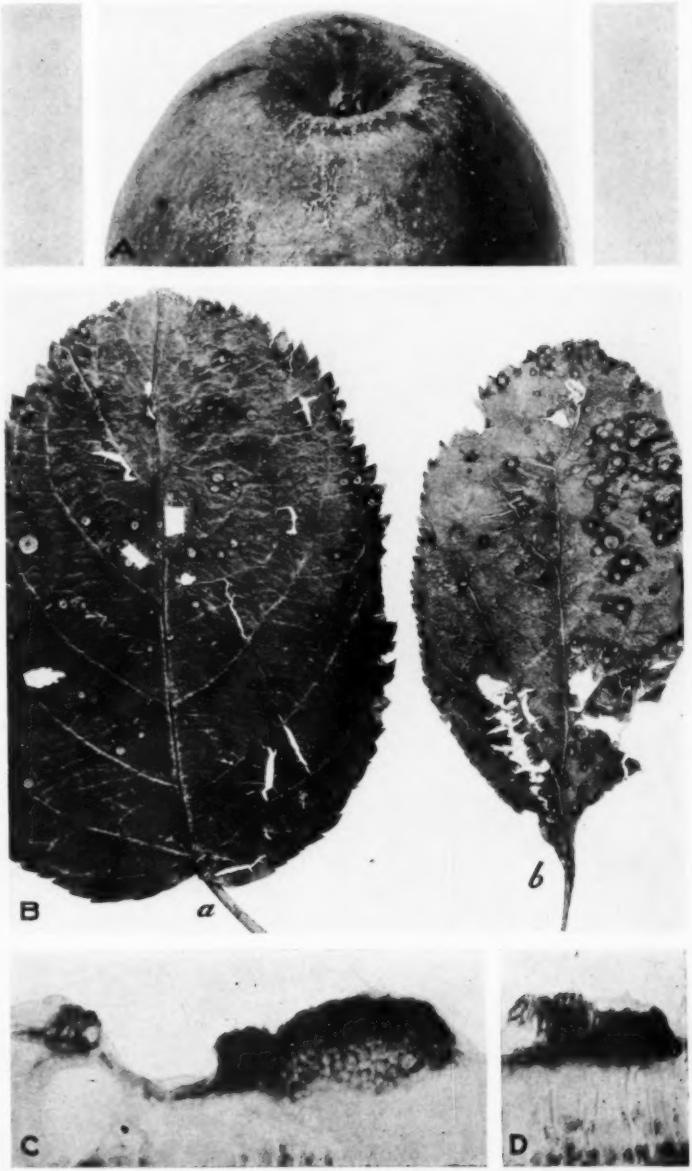
of those described for this fungus as referred to later in this paper. (Pp. 4, 5.) In addition, on one of the Italian specimens there was seen a 3-cell elongate colored body interpreted as possibly a greatly swollen conidium of this fungus. Hyaline conidia of the type illustrated in Plate 3, E and F, some of which were biguttulate, were produced in great abundance within an 18-hour period, when small masses of a young culture on potato-dextrose agar were transplanted to a corn-meal poured plate to which a few drops of sterile water

[5] Color reading by J. Marion Shull based on the following publication: RIDGWAY, R. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 p., illus. Washington, D. C. 1912.

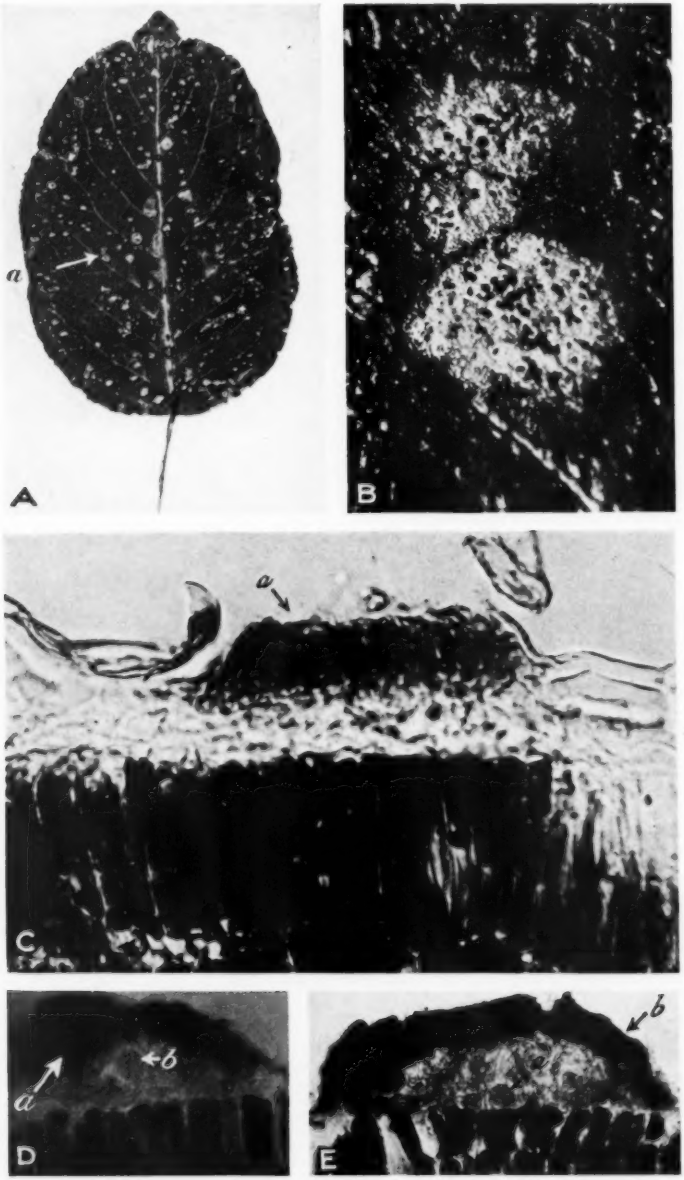
[6] Op. cit. (See footnote 3.)

[7] BRIOSI, G., and CAVARA, F. I FUNGHI PARASSITI DELLE PIANTE COLTIVATE OD UTILI. Fasc. 11, No. 274. Pavia, 1896. This material, labeled "*Hadrotrichum populi* Sacc.,"[8] consists of three specimens for which the following data are given: "Estate 1894—Sul Pero—Avellino— D. Peglion; id. 1891—id.—Orto botanico di Pavia; id. 1890—Sul Melo—id. id."

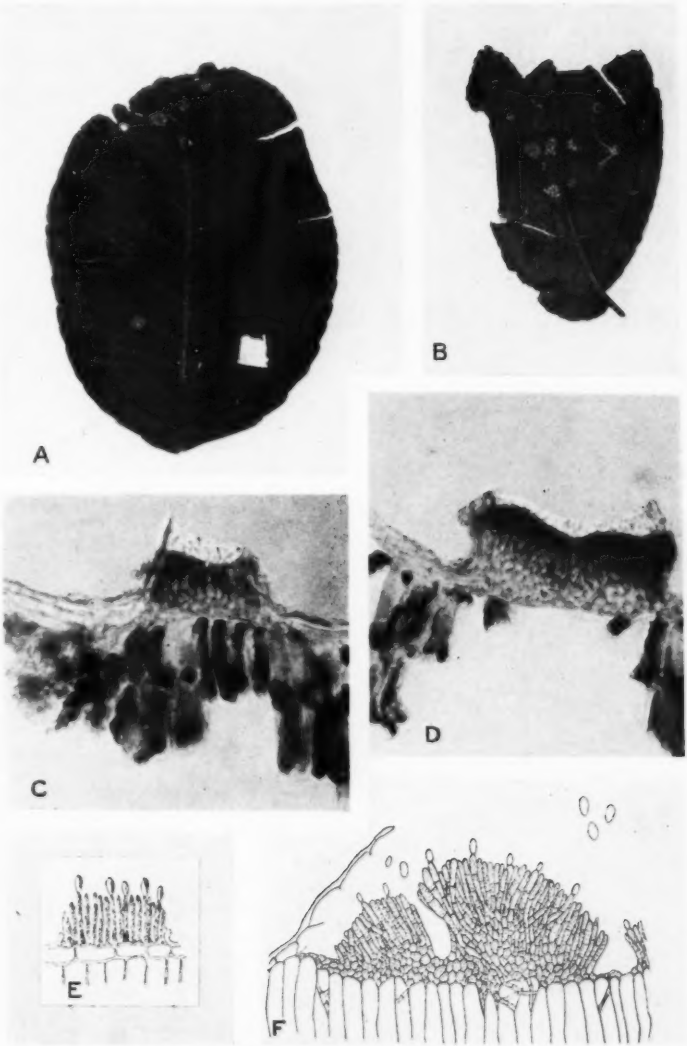
[8] This species was originally described on black poplar (*Populus nigra* L.) as doubtfully of the genus Hadrotrichum, and the binomial was therefore written as *H.? populi* (*43, p. 264*). As in this instance, where the question mark has been dropped from this name in literature cited, it is also omitted in the present paper.



A, Part of Jonathan apple fruit illustrated by Zschokke, showing lesions apparently of apple and pear anthracnose (× 1); B, *a* and *b*, general appearance of anthracnose lesions on upper surface of apple leaves (× 1); C and D, sections of conidial fructifications on upper surface of apple leaves (× 380). Material (B–D) from Woronichin, Transcaucasia, Russia, in 1913. Ex Herb. Inst. Mycol. and Phytopath. Leningrad. Photographs (A, C, and D) by J. F. Brewer and (B) by W. R. Fisher



Upper surface of pear leaves infected by *Plectodiscella piri:* A, General appearance of lesions (× 1); B (enlargement of A, *a*), fructifications of both stages of the fungus (× 22); C–E, sections through fructifications represented in B; C, *a*, an acervulus; D and E, ascomata, with conidiophores at one side (*a*) of D; D, *b*, and E, *a*, asci; E, *b*, epithecium or dark stroma, covering ascoma. C, × about 600; D and E, × 360. Material from same source as that shown in Plate 1, B–D. Photographs by J. F. Brewer



Plectodiscella piri: A–D, On pear leaves from Italy, identified by Briosi and Cavara as *Hadrotrichum populi* Sacc. and distributed by them as No. 274, I FUNGHI PARASSITI DELLE PIANTE COLTIVATE OD UTILI. Fasc. 11. Pavia, 1896. One of these leaf specimens of the fungus was collected at Avellino in 1894 by Peglion and is typical of his *Gloeosporium pirinum;* the other was collected in 1891, at Pavia. A, Upper leaf surface (× 1); B, lower leaf surface (× 1); C and D, sections of acervuli on lesions on upper surface of A and of B, respectively (× 380). Photographs by J. F. Brewer. E, On pear leaves, as illustrated by Ferraris (19, fig. 174, I), under the name of *Hadrotrichum populi.* F, On apple leaves, as illustrated by Maublanc (35, Figs. XIII and XIV), under the name of *Melanobasidium mali*

had been added. Some conidia were already present in the culture from which the transfers were made. Granular masses in some sections of lesions on apple fruits were interpreted as possibly consisting of microconidia.

Plates 1, C and D, and 2, C, *a*, represent the conidial stage of the fungus; Plate 2, D, both the conidial stage (*a*) and the perfect stage (*b*); and Plate 2, E, only the perfect stage. Woronichin's (*55, fig. 3, and pl. 1, fig. 1*) illustrations of dark hyphae at the apex of a young pustule of this fungus, as well as of converging hyphae at the center of another pustule on the same material, are interpreted by the present writer as conidiophores of *Plectodiscella piri*. These structures, however, together with such dark hyphae or stroma covering the ascoma as are here shown in Plate 2, E, *b*, are termed by Woronichin (*55*) the "epithecium" or "shield." The dark hyphae or stroma are believed to have formed entirely or almost entirely within the epidermis, as is illustrated for a closely related fungus on Lima bean (*Phaseolus lunatus macrocarpus* Benth.), tentatively identified as *Elsinoe canavaliae* Rac. (*30, pl. 4, C, c, and pl. 5, D and K, b*). The Elsinoe structure illustrated in Plate 2, C, *a*, of the paper just cited seems to be a development homologous to what Woronichin (*55, pl. 1, fig. 6*) described in *Plectodiscella piri* as a small, cup-shaped, almost superficial ascoma practically inclosed in a coat of brown cells. In both instances the darkening of the hyphae is possibly due to oxidation following exposure, or, as explained by Woronichin (*55*) in the case of the apple and pear fungus, to the pronounced isolation of the ascoma from the substratum.

It seems that since his report of the perfect stage of *Plectodiscella piri*, Woronichin (*56, p. 221*) has identified its conidial stage as *Hadrotrichum pirinum* (Pegl.) Sacc., which, as will be explained later, actually represents the conidial stage of this ascomycete. This identification by Woronichin was made in reporting a leaf spot on pear growing in a Caucasian nursery. His description of the lesions and of the disposition of pustules of the pathogene thereon is essentially the same as that given in connection with his (*55*) description of the perfect stage, i. e., *Plectodiscella piri*. The conidia are described as 4μ to 5μ in diameter (*56, p. 221*).

TAXONOMY

As stated earlier in this paper, Jenkins and Horsfall's (*31*) discovery of the imperfect stage of *Plectodiscella piri* was based upon conidia produced in a subculture of an isolation by Osterwalder and upon those found on the specimens from Transcaucasia on which Woronichin (*55*) had discovered the perfect stage. The recent finding of a Sphaceloma on an apple fruit from Italy (*28*) at once suggested that the fungus had long been known to Italian mycologists. The certainty of this is now established through the specimens from Italy identified by Briosi and Cavara as *Hadrotrichum populi* Sacc.[9] The fungus is here unquestionably of the genus Sphaceloma, and a comparison of it with typical material of *Plectodiscella piri* [10] shows that

[9] Op. cit. (See footnote 7.)
[10] Op. cit. (See footnote 3.)

it is this species. The Italian material represents the fungus on pear leaves from Avellino and Pavia (pl. 3, A-D) and on an apple leaf from Pavia. That on the leaf from Avellino is from the collection of *Gloeosporium pirinum* on which Peglion (*39*) based his description of that fungus.

The disease caused by *Gloeosporium pirinum* was termed "pear anthracnose" by Peglion (*39; 40, p. 267*), and this name was later employed by Briosi (*13, p. 365; 14, p. 70*) in reporting the disease on pear. In the present paper the disease is referred to as anthracnose of both apple and pear. Peglion (*39; 40, p. 268*) observed the similarity of the leaf lesions produced by this disease to those of grape anthracnose on leaves of grape (Vitis). He (*39; 40, p. 268*) made some notations on the susceptibility of pear varieties to infection by *Gloeosporium pirinum*. Recently he has written that this fungus is common in the region of Bologna, where he is now located.

Saccardo (*44, p. 136*), in 1915, reporting the fungus on this host in France, made the new combination *Hadrotrichum pirinum* (Pegl.), for which he gave the synonyms *Gloeosporium pirinum* Pegl. (*39, p. 4*), *Hadrotrichum piri* Montem. (*35, p. 226*), and *H. populi* Sacc. var. *piri* (Montem.) Ferr. (*19, p. 875*). On the basis of Maublanc's technical description (*34, p. 70*) and illustrations (*34, figs. xiii and xiv*) of *Melanobasidium mali* Maub. and an examination of a fragment of the specimen on which the description is based,[11] this fungus is here identified as the same as *Gloeosporium pirinum*, or *Plectodiscella piri*.

The reports of *Hadrotrichum pirinum* from France and of *Melanobasidium mali* from Spain constitute the only records at hand of *Plectodiscella piri* in these countries. Reference to its occurrence in Portugal was made in the historical section of this paper.

The illustrations of this fungus by Ferraris (*20, fig. 174, I*), under the name of *Hadrotrichum populi*, and by Maublanc (*34, figs. xiii and xiv*), under that of *Melanobasidium mali*, are reproduced in Plate 3, E and F, respectively, while the original technical descriptions of *Gloeosporium pirinum*, *Hadrotrichum piri*, *Melanobasidium*, and *M. mali*, typifying this genus, follow:

GLOEOSPORIUM PIRINUM Pegl.

Maculis initio punctiformibus, rubro-cinctis, inde effusis, rotundis, saepe confluentibus, ad centrum griseis, vel sordide brunneis amphigenis; acervulis minutis 150–300μ diam. erumpentibus, olivaceo-chlorinis; conidiis ovatis vel subcylindraceis, continuis, eguttulatis, 6–4, hyalinis; basidiis bacillaribus, 20–25 $\times$4, minute granulosis hyalinis vel dilute fumosis.

Hab. in foliis *Piri communis* prope Avellino—It. austr. —Vere 1894.

MELANOBASIDIUM nov. gen. (Tuberculariées Dématiées).

Foliicolum, maculicolum; sporodochia minima, erumpentia, atra, ex hyphis ramosis, septatis, intricatis composita, sporophoris cylindricis, densis, septatis, concoloribus vestita; conidia solitaria, acrogena, ovoidea, hyalina.

MELANOBASIDIUM MALI nov. sp.

Maculis albidis, ovoideis vel elongatis, margine brunnea, angusta cinctis; sporodochiis punctiformibus, epiphyllis, demum epidermide fissa superficialibus, 170–190μ latis; conidiis ovoidiis, hyalinis, 4.5–5.5$\times$2.5–3μ.

In foliis vivis *Piri Mali* ad Sevillam, Hispaniæ.

[11] "*Melanobasidium mali* Maublanc sur *Pirus malus* Seville 1900" (fragment of type). Ex Herbarium, Station Centrale de Pathologie Végétale, Ministère de l'Agriculture, Institut de Recherches Agronomiques, Versailles, France. Specimen received through the courtesy of G. Arnaud.

Cette espèce est nettement parasite sur les feuilles du Pommier, où elle forme de petites taches blanches, bordées d'une marge subérisée. Je n'ai pas de renseignements sur l'extension de ce champignon et les spores de l'unique échantillon reçu n'ont pas germé.

HADROTRICHUM PIRI.

Acervulis puntiformibus, atris, in maculis foliarum dealbatis, epiphyllis, subsuperficialibus: strato proligero subcuticolare, e cellulis oblongis dense stipatis fuligineis conflato; conidiis globoso-ovoidies (4–5×4μ), olivaceo-fuligineis.

Hab. In foliis vivis *Piri communis*, Montubeccaria (prov. Pavia).

A few years before Peglion (*39, p. 6*) described *Gloeosporium pirinum*, Cavara (*17, p. 184*), reporting the occurrence of *Hadrotrichum populi* on *Populus nigra* L. in Lombardy, Italy, stated that a closely allied form affects "*Rosa* esp. cult." and "*Rubus corifolius*." He (*18, p. 282*) later asserted that this Hadrotrichum species affects "Rosa, Rubus, Sorbus" and "Pyrus." At about the same time Briosi reported in Italy *Hadrotrichum* sp. on wild rose (*7, p. xviii*), and *H. populi* (*8, p. xix*) and *H. populi* "forma del pero" (*9, p. xxvi*) on pear. He continued to refer to the pear fungus either as *H. populi* (*10, p. 298; 12, p. 663; 14, p. 70*) or as *Gloeosporium pirinum* (*13, p. 365*). He also reported *H. populi* on rose in Meaux, France (*10, p. 313*), and on maple (*Acer negundo* L.) (*11, p. 541*) in Italy. Ferraris (*20, p. 875*) included European mountain ash (*Pyrus aucuparia* Ehrh.) among the hosts for *H. populi* var. *piri*.

Even as late as 1920 Montemartini (*36, p. 122*) was apparently unaware of the nomenclatorial status of his *Hadrotrichum piri*, for, citing only Cavara (*18*), he recognized this binomial and *Gloeosporium pirinum* as synonyms of *H. populi*. At the same time he reported what he interpreted to be this fungus on pomegranate (*Punica granatum* L.) growing at Montubeccaria, Pavia, the type locality for *H. piri*.

More recently, Borg (*5, p. 238–239*) and Marchionatto (*33, p. 10–11*) have reported *Gloeosporium pirinum* on pear in the island of Malta and in Argentina, South America, respectively; while in the Crimea, Russia, Garbowski (*21, p. 255–256*) has identified *Hadrotrichum populi* on apple, pear, plum (*Prunus domestica* L.), poplar (*Populus nigra*), and white beamtree (*Sorbus aria* Crantz), and, as previously noted, in the latter country Woronichin (*56, p. 221*) has reported *H. pirinum* on pear in the Caucasus and *Plectodiscella piri* on both apple and pear in Transcaucasia (*55*).

Garbowski (*21, p. 256*) stated that in the Crimea *Hadrotrichum populi* causes considerable damage to fruit trees, particularly apple; he observed it on both leaves and fruits of several different varieties. He described the fruit spot as circular, with a whitish central part, and a reddish-brown border. Reaching a centimeter in diameter, the whitish area was dotted with the dark pustules of the fungus, which had ruptured the dead epidermis. He noted also the close resemblance of the fruit spot to that caused by *Phoma pomorum* Thuem., and stated that it is probable that infection due to the Hadrotrichum is often ascribed to this fungus.

Specimens of the so-called Hadrotrichum on apple leaves from the Crimea, as well as of *Gloeosporium pirinum* on pear from Argentina, recently received through the courtesy of Garbowski and Marchionatto, respectively, are of the same general appearance as those of

Plectodiscella piri from other sources, which have been examined by the writer.

Marchionatto (*33, p. 11*), reporting the fungus only on leaves of pear, stated that it was fairly widespread in the Province of Buenos Aires and the islands of Delta del Parma. His advice (*33, p. 11*) for the control of the disease is similar to that given by Peglion (*39; 40, p. 269*).

The reports of Hadrotrichum on rose presumably concern the Sphaceloma on rose; those on brambles, *Plectodiscella veneta* (Speg.) Burk., or the perfect stage of *Gloeosporium venetum* Speg. (*16*); and those of Hadrotrichum and *G. pirinum* on apple and pear, *P. piri*. Although these three similar fungi, occurring on rosaceous hosts, and *Hadrotrichum ? populi*, on poplar, have been treated as comprising one and the same organism, it seems advisable to keep them, tentatively at least, as separate species, as originally described. It has been mentioned elsewhere by the present writer[12] that Alexander (*1, p. 72*) reported infection of apple fruits by *P. veneta*, but that he did not furnish absolute proof of such pathogenicity. This species and the other two from rosaceous hosts just mentioned were separable, as far as compared by the writer,[12] but this is not interpreted necessarily to mean that each is entitled to the rank of species.

Lindau (*32, p. 684*), in referring to the doubtful classification of *Hadrotrichum populi* in the genus Hadrotrichum (*43, p. 264*), suggested that the species be retained there until it could be investigated further. An examination of typical material of the fungus on *Populus nigra*,[13] on which, as previously stated, it was originally described, as well as of Briosi and Cavara's[14] illustration of it, shows that it possesses the characteristics of the genus Sphaceloma and that it is possibly a distinct species. Therefore it is here transferred to the genus Sphaceloma as *S. populi* (Sacc.), n. comb. The fungus is reported not only on black poplar, but also on Lombardy poplar (*P. nigra italica* DuRoi) in Italy[15] and South America (*46, p. 192*) and on "*Pioppo canadense*" (*53, p. 305*) in Italy.

Isolations of Sphaceloma from pear, poplar, or strawberry tree, or tests to determine whether the Sphaceloma on apple will infect pear, and that on pear apple, seem not to have been made; nor has there been available fresh material of the pear fungus or any specimen of what may prove to be Sphaceloma on maple, pomegranate, plum, European mountain-ash, or white beamtree.

Brizi (*15*) has reported an anthracnose of almond (*Amygdalus communis* L.) in Italy, which he regards as similar to anthracnose of grape; also Von Höhnel (*26, p. 65–67*) has added a species to Maublanc's genus Melanobasidium, but its characteristics as described (*26, p. 65–67*) may exclude it from this genus.

Before erecting the ascomycetous genus Plectodiscella and the family Plectodiscellaceae for the apple and pear anthracnose organism, Woronichin (*55*) considered placing the species in one of several genera already described with each of which it has characteristics in common.

[12] JENKINS, A. E. ROSE ANTHRACNOSE CAUSED BY SPHACELOMA. (Unpublished manuscript.)
[13] Selva, Italy, October, 1877. (SACCARDO, P. A. MYCOTHECA VENETA, Century 8-12 (pr. p.) No. 1256.) (Cited in *43, p. 264.*)
[14] BRIOSI, G., and CAVARA, F. I FUNGHI PARASSITI DELLE PIANTE COLTIVATE OD UTILI. Fasc. 13–14, No. 139. Pavia, 1900.
[15] Op. cit. (See footnote 14.)

One of the genera considered was Elsinoe, typified by *E. canavaliae* and originally reported on *Canavalia gladiata* (Jacq.) DC. According to Woronichin (*55*) the ascoma in *Plectodiscella piri* develops intraepidermally, whereas in *Elsinoe canavaliae* it forms subepidermally; furthermore, lesions of scab of Canavalia are thickened, while in apple and pear anthracnose such lesions are not found. The two species can not be separated by these criteria, for intraepidermal as well as subepidermal ascomata occur in the Lima-bean fungus tentatively identified as *E. canavaliae*; furthermore, data at present available show that lesions resulting from attack by Sphaceloma may be not only of normal thickness, but also of increased or (*3, 27*) even of less than normal thickness. The fact that hyperplastic lesions occur in the anthracnose of brambles (*16, 49*) and of rose[16] suggests that they may be found in the apple and pear disease.

Woronichin (*55*) found resemblances between the perfect stage of *Plectodiscella piri* and Molleriella Wint. (*54, p. 102*) not Moeleriella Bres. (*6, p. 292*). The latter genus at the time his (*55*) paper was written had been removed (*25, p. 349*) from the family Myriangiacei Nyl. (*37, p. 139*) and then transferred (*25, p. 349*) to the discomycetous family Agyriaceae Von Höhn. (*25, p. 362*). If more information had been available concerning Molleriella it is probable that Woronichin (*55*) would have classified his new fungus in this genus, which is older than Elsinoe. He (*55*) compared it with the myriangioid genera Ascostratum Syd. and Kusanoa P. Henn., as well as with Elsinoe and Myriangina (P. Henn.) Von Höhn. (*25, p. 372–373*); of these two it resembled Elsinoe more than Myriangina. Myriangina was originally classified by Hennings (*24, p. 55*) as a subgenus of Myriangium Mont. and Berk. (*4*), on which is founded the Myriangiaceae. Upon removing Myriangina from this family, Von Höhnel (*25, p. 373*) erected for this genus, as well as for Elsinoe until then classified in the Exoascaceae (*42*), the family Elsinoeaceae Von Höhn. He was uncertain of its systematic position, but suggested that its affinities might be with the Plectodiscales or with the Protodiscales. Woronichin (*55*) concluded to place his new family Plectodiscellaceae between the Elsinoeaceae and the Discomycetes, explaining that it was undoubtedly related to Molleriella, and through Ascostratum and Kusanoa to the Myriangiaceae.

Soon afterwards the Myriangiaceae were treated as an order by Theissen (*50, p. 311*), i. e., as the Myriangiales Starb. (*42*), and the families Elsinoeaceae and Plectodiscellaceae were placed in this order by Theissen and Sydow (*51, p. 437*). For these families they created the suborder Protomyriangieae and distinguished them by the presence of an epithecium or shield in the Plectodiscellaceae and its absence in the Elsinoeaceae. It has been shown earlier in the present paper that this is not a valid criterion for the separation of the type species of the genera Elsinoe and Plectodiscella; it follows, therefore, that it is not a valid criterion for the separation of the two genera or of the families created for them. In 1925 Arnaud (*2*) merged the two families and transferred Elsinoe, Plectodiscella, and Myriangina to Hennings's (*22*) genus Uleomyces. Uleomyces, Myriangium, Kusanoa, Ascostratum, and a few others fall in the Eumyriangieae of Theissen

[16] JENKINS, A. E. Op. cit. (See footnote 12.)

and Sydow (*51*), the only other suborder in the Myriangiales as classified by them (*51*). Arnaud, who (*2*) explains that the order is only imperfectly known, thus seems to disregard the two suborders of Theissen and Sydow. As a matter of fact, in the orientation of their stroma, Myriangina, as discussed by the writer (*29*) and by others, and some other related genera (*48*) actually partake of both suborders.

Arnaud (*2, p. 678*) transferred *Plectodiscella piri* and *P. veneta* to what he termed "the Elsinoe section" (*2, p. 688*) of the genus Uleomyces. Shear (*45*) did not agree with this transfer, because "Uleomyces has a superficial stroma and many-septate, muriform, brown ascospores, instead of 3-septate hyaline spores as in the type of Plectodiscella." Citing Arnaud's (*2*) account of *Elsinoe canavaliae*, Shear (*45*) stated:

> It is very clear that Elsinoe is a synonym of Plectodiscella, unless [here apparently following Theissen and Sydow (*51*) and not Woronichin (*55*)] the dark-colored superficial cover in Elsinoe be considered a distinctive character * * *.

These statements were made by Shear (*45*) in connection with his report of *Elsinoe ampelina* (D By.) Shear, recently found by him in the United States, the fungus being possibly the same as *E. viticola* Rac. (*31, 45*), originally reported from Java.

As previously mentioned in the present paper, the writer (*30*) has shown that a superficial cover like that in Plectodiscella is found in Elsinoe on Lima bean. In other respects also the two genera are identical. Colored muriform spores occur in *Elsinoe canavaliae* (*30*), so that in this particular Elsinoe, or Plectodiscella, agrees with Uleomyces. Colored ascospores occur also in the genus Myriangium, as already reported by Petch (*41, p. 62-63*). Had this characteristic of Myriangium been known to Hennings, according to his own statement, he (*23*) would have treated his genus Uleomyces as a synonym of Myriangium. Theissen (*50, p. 312*) and Theissen and Sydow (*51, p. 439*), on the other hand, separate Uleomyces and Myriangium on the basis of the distribution of asci in the ascoma; i. e., in Uleomyces the asci occur throughout the ascoma, whereas in Myriangium the lower part of the ascoma is sterile. Arnaud (*2*) recognizes the same distinction. Of these two genera only Myriangium has been cultured by the present writer, no living material of Uleomyces being available. It is evident that further investigation of Uleomyces, including a study of its growth in culture, is essential to a satisfactory comparison of this genus with the others just mentioned.

As previously stated, Woronichin (*55*) has suggested that Plectodiscella may be identical with Molleriella. It appears to be more closely related to this genus than to Uleomyces, but actual material of Molleriella is not available with which to make a direct comparison. The genus, which is older than Uleomyces, is included in the Myriangiales by Arnaud (*2*).

It is convenient here to treat Elsinoe as a distinct genus, with Plectodiscella and Melanobasidium as synonyms. *Plectodiscella piri* and *P. veneta* are referred to Elsinoe as *E. piri* (Wor.), n. comb., and *E. veneta* (Speg.), n. comb. In addition to *P. piri* and *Uleomyces piri*, synonyms of *E. piri* are, of course, *Gloeosporium pirinum*, *Hadrotrichum piri*, *H. populi* var. *piri*, *H. pirinum*, and *Melanobasidium mali*.

SUMMARY

Plectodiscella piri Wor., which causes apple and pear anthracnose, a disease of considerable economic importance, is widely distributed in Europe. It occurs also in South America, but is not known to be established in North America, although it has recently been intercepted at ports of entry in the United States.

The morphology, taxonomy, and history of the fungus are discussed. Structures termed the "epithecium" or "shield" by Woronichin in describing its perfect stage are interpreted as the conidiophores of its Sphaceloma or conidial stage. The data presented show that Woronichin's reasons for not placing this fungus in the genus Elsinoe are invalid, as is also Theissen and Sydow's basis for separating the families Elsinoeaceae and Plectodiscellaceae. Although Arnaud has transferred Plectodiscella to the genus Uleomyces, with consequent nomenclatorial changes, it is suggested that Uleomyces be investigated further before this transfer is accepted. Elsinoe, which Arnaud also treats as a synonym of Uleomyces, is here regarded as a distinct genus, and Plectodiscella and also Melanobasidium are considered as synonyms; *Plectodiscella piri* and *P. veneta* are referred to Elsinoe. *Melanobasidium mali*, the type of Melanobasidium, is regarded as a synonym of *Elsinoe piri*.

Hadrotrichum populi, on poplar, is transferred to the genus Sphaceloma.

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CANKER OF ASH TREES PRODUCED BY A VARIETY OF THE OLIVE-TUBERCLE ORGANISM, BACTERIUM SAVASTANOI [1]

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INTRODUCTION

A canker of the European ash tree, *Fraxinus excelsior* L., occurs in England, Austria, Germany, France, and Italy. It is a stem disease of the canker type that progresses slowly from year to year, involving the bark and wood and stimulating the growth of the stem at the cankered areas. The disease has not been reported in this country. In 1922 the writer found what appeared to be bacterial cankers on a European ash tree in one of the parks in Washington, D. C. The branches of the tree were badly distorted with cankers, and the tree itself was nearly dead and was being cut down at the time. Some of the cankers were secured and from them numerous isolations were made, but no pathogenic organism similar to that obtained from European cankers could be isolated.

REVIEW OF LITERATURE

Noack (*2*)[2] in 1893 described and pictured cankers on *Fraxinus excelsior* occurring in Germany. He saw bacteria while studying cross sections through the cankers, and concluded that the disease was a bacterial one; but he did not isolate the organism or produce the disease experimentally.

Vuillemin (*8*) claimed that the ash canker in France was the same as the olive-tubercle disease, but he did not give his reasons. He may have reached this conclusion because of the fact that the ash tree belongs to the same natural family as the olive; but there is no regular tubercle or knotlike appearance in the ash disease.

Massee (*1, p. 520*) described the ash canker as a distinct bacterial disease:

> The 4- or 5-year-old stems or branches of young ash trees are frequently disfigured by the presence of cankered spots, varying in size from small cracks with thickened margins, half an inch long, up to rugged patches forming irregular cavities in the wood, and bounded by irregular outgrowths of callus, which may extend for several inches.

In a detailed account of the olive-tubercle disease, with a description of the olive-tubercle organism, *Bacterium savastanoi* E. F. Smith, Smith (*6*) included a paragraph about the ash-canker disease, stating that cankered stems had been sent to the United States from Europe and that from them the present writer had isolated an infectious organism that reproduced typical cankers on both American and European ash trees. At that time and for some years previously

[1] Received for publication Dec. 1, 1931; issued May, 1932.
[2] Reference is made by number (italic) to Literature Cited, p. 722.

Smith had been greatly interested in the olive-tubercle disease. He had investigated the disease and its causal organism, as well as the early literature connected with the subject (*4, 5, 7*). Although aware of the relationship of the olive and ash trees, Smith did not agree with Vuillemin (*8*) in the theory that both the olive-tubercle and the ash-canker disease were produced by the same bacterium. This difference of opinion, as well as the continued appearance of the olive-tubercle disease in the olive groves of California and the discrepancies in the tests of the Italian organism isolated from the olive tubercle by Petri and described by him (*3*), influenced Smith to send ash-canker material from Vienna and later olive tubercles from Portofino, Italy, to the writer in Washington, D. C.

THE ASH-CANKER DISEASE

It was while Smith was in Europe in 1913 that he found ash trees badly diseased with cankers. One clump of trees, numbering about 40, in the neighborhood of Vienna, had more or less of the disease on both trunks and branches. Smith believed that by means of fresh isolations from the young ash cankers and from young olive tubercles the differences between the two organisms could be readily determined. Experiments carried out by the writer with both the Austrian and the Italian material convinced Smith that the ash-canker organism was a variety of the olive-tubercle organism, *Bacterium savastanoi*, and not a distinct species as he had supposed.

Material showing various stages of the ash canker was received in Washington a little more than a month after it had been sent from Austria by Smith. (Fig. 1, A and B.) The earliest stages showed a longitudinal split in the bark, from a few millimeters to a centimeter in length, with darkened raised tissue inside the split. The older stages showed a spreading out of this split, from which darkened corky tissue protruded. The disease did not often extend very far into the wood, the thickened portion being mostly bark; but the growth of the bark and wood was greatly stimulated, the diameter of the cankered area being often twice that of the stem above or below the canker. The largest cankers on the trees had not been secured; those received varied from 5 mm to 8 cm in length and from 3 mm to 5 or 6 cm in width. One stem 2.5 cm in diameter was almost encircled by cankers.

The disease disfigures the tree and destroys its commercial value. Noack (*2*) found instances in which leaves and leafstalks were infected. No diseased leaves were received by the writer, but since leaf infection of the olive tree is a common condition in the olive-tubercle disease, Noack is probably correct in his belief that infection may begin in leaves and leafstalks of the ash and spread to the stems.

Cross sections through the diseased portions of the cankers received from Vienna did not show bacteria in pockets like those in the olive stem. A white gummy substance that oozed from the sections was always present. This substance was filled with nonmotile particles which looked like bacteria but which because of their density could not be identified with absolute certainty. As the specimens were cut a long time before they were received, the condition of the tissues was somewhat changed and the bacteria were inactive.

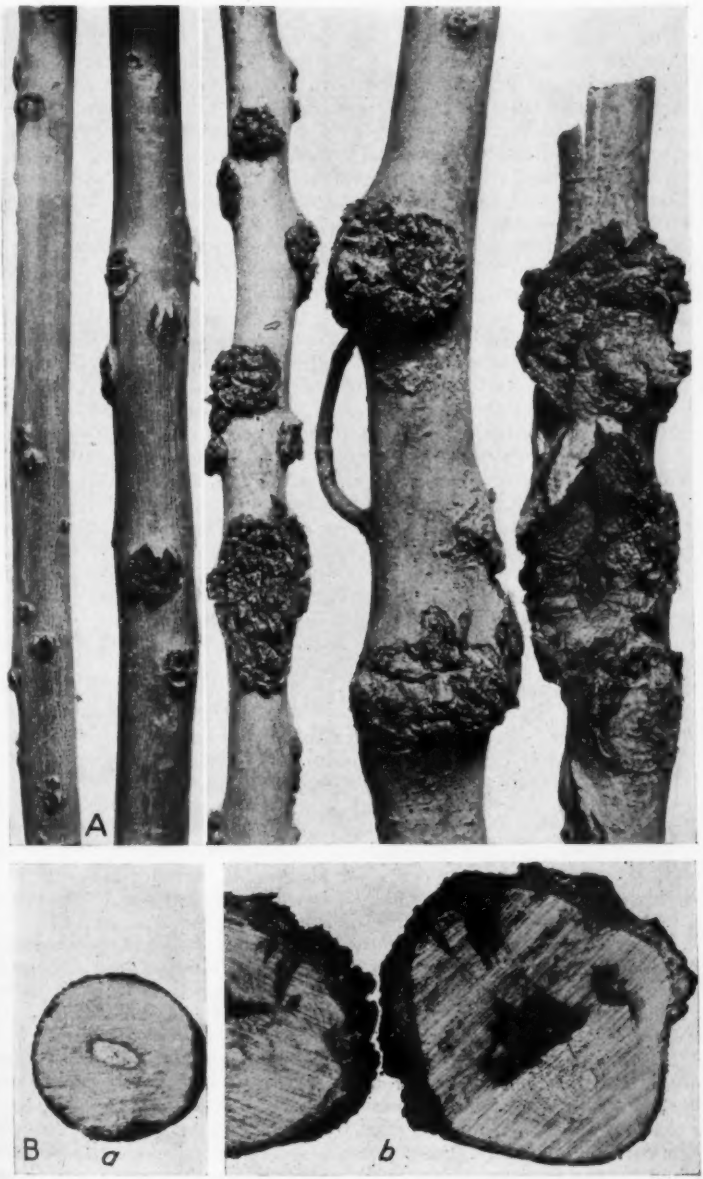

FIGURE 1.—A, Ash canker from Vienna, Austria, in various stages of development. Photographed on arrival, December 8, 1913. × ½. B, Cross sections through ash stems; *a*, healthy; *b*, diseased. Natural size

ISOLATIONS AND INOCULATIONS

Numerous sets of plates were poured, but no common colony appeared, probably because of the dry condition of the material. Various colonies were picked off and inoculations made into young American ash trees, *Fraxinus americana* L. Of nine colonies used, three proved to be infectious. Two of these are shown in Figure 2, A and B. The control punctures produced no outgrowth. (Fig. 2, C and D.) The three colonies were alike in macroscopic appearance; all were white and resembled colonies of the olive-tubercle organism (*Bacterium savastanoi*).

European ash trees were inoculated with very satisfactory results. (Fig. 3; fig. 5, C.) The inoculum used was a subculture from an agar poured-plate colony. The infection took place more rapidly than on the American ash, and larger cankers, like the original ones from Austria, developed. In less than a week water-soaked areas showed around the inoculation pricks. A blisterlike swelling followed; then a split occurred. Later the curled bark and the roughened swellings of wood just under the bark extended for some distance out from the split. In five months cankers 2.5 cm long had formed. The splitting and darkening, with accompanying protuberances, continued to develop for a year or more until a large canker was produced.

In the spring the tender shoots of olive trees in the greenhouse were inoculated with subcultures of the original ash-canker organism. The shoots were watched far into the summer, but no well-defined canker or any indication of a tubercle appeared. Because of a slight disturbance in the tissue of the olive stem, it was thought for a time that cankers were forming, but the development did not continue. Inoculations on the olive were repeated twice with negative results. (Fig. 4, A and B.)

Inoculations with an actively infectious strain of *Bacterium savastanoi*, isolated by the writer from olive tubercles received from Portofino, Italy, were made into both European and American ash in four different tests, but neither canker nor tubercle was produced. Photographs of ash stems, some inoculated with the olive-tubercle organism and others with the ash-canker organism, are shown in Figure 5, A and B.

The virulence of this isolation of *Bacterium savastanoi* had been previously established by repeated successful inoculations and was now tested again on olive trees. In two months knots 2 to 2.5 cm in diameter had grown at the inoculated places. (Fig. 4, C and D.)

The ash-canker organism was reisolated from the cankers produced on the inoculated European ash trees, and this reisolated organism likewise produced typical cankers when reinoculated into other European ash trees. (Fig. 6, B, and fig. 7). Control punctures are shown in Figure 6, A.

The cankers produced by inoculations were studied in cross sections stained and unstained, as the original European material, which was received a long time after it was cut, had been found unsatisfactory for section study. The bacteria were abundant between the cells of the cork and the bark parenchyma and also in a slime in the cavities formed by the disintegration of the cells. Here they were seen to be motile. There were no pockets, however, as in the olive tubercle. That bacteria were present in the wood also was proved by isola-

FIGURE 2.—American ash stems inoculated with *Bacterium sarastanoi* var. *frazini* isolated from European ash canker from Vienna, Austria: A and B, Inoculated May 1, 1914, with two different colonies, of which B was the more infectious, and photographed August 10, 1914: C and D, control punctures on American ash stems made on the same date. About natural size

FIGURE 3.—European ash stems inoculated with ash-canker organism May 1, 1915, more than a year after isolation from European material. Photographed October 19, 1915. × nearly 2

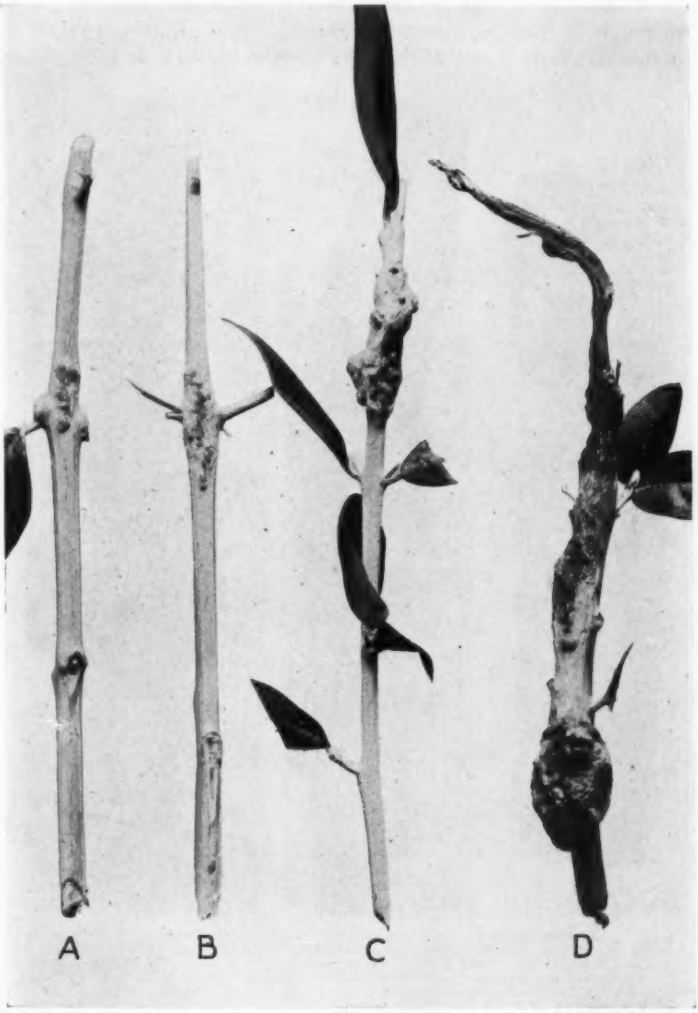

FIGURE 4.—A and B, Olive stems inoculated with the ash-canker organism October 31, 1914, and photographed February 18, 1915; C and D, olive stems inoculated with the olive-tubercle organism December 4, 1914, and photographed February 18, 1915. All natural size

tions, as the organisms could not with certainty be determined microscopically.

LABORATORY TESTS AND COMPARISONS OF ASH-CANKER AND OLIVE-TUBERCLE ORGANISMS

Although *Bacterium savastanoi* and the ash-canker organism did not cross-inoculate in any of the experiments, cultural and morpholog-

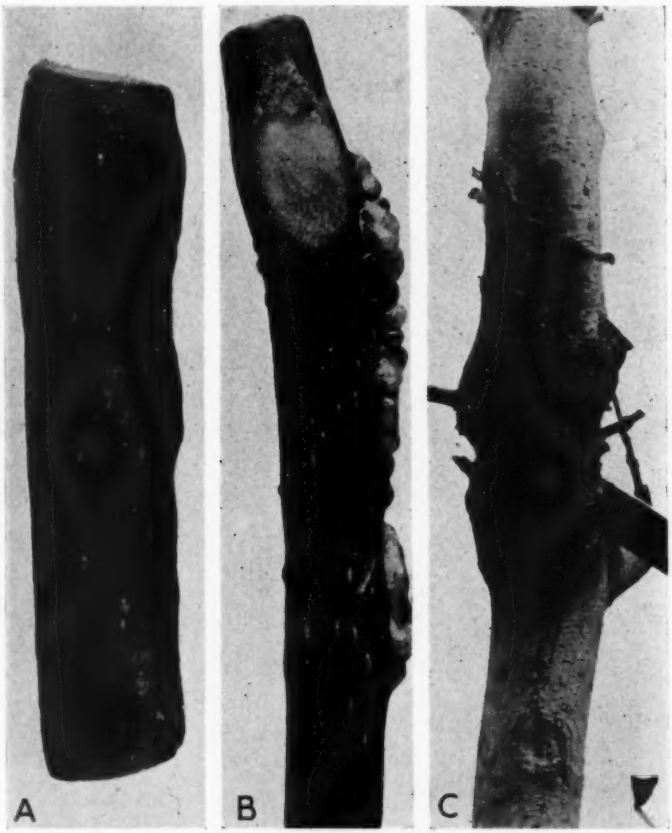

FIGURE 5.—European ash stems inoculated with the olive-tubercle organism and the ash-canker organism. A, Stem inoculated with the olive-tubercle organism April 30, 1915, and photographed May 12, 1915. Neither tubercles nor cankers formed. × 5. B, Stem inoculated with the ash-canker organism April 30, 1915, and photographed May 12, 1915. × 5. C, Stem inoculated with the ash-canker organism May 7, 1915, and photographed October 10, 1918. Natural size

ical tests showed them to possess a marked degree of similarity, and it is thought that they must be closely related species or varieties of the same organism exhibiting individual host tendencies. Comparisons were made, not only with the usual laboratory tests but also with the tests which Petri (*3*) used in his study of *Bacterium savastanoi* and which he published in 1909. (Tables 1 and 2.)

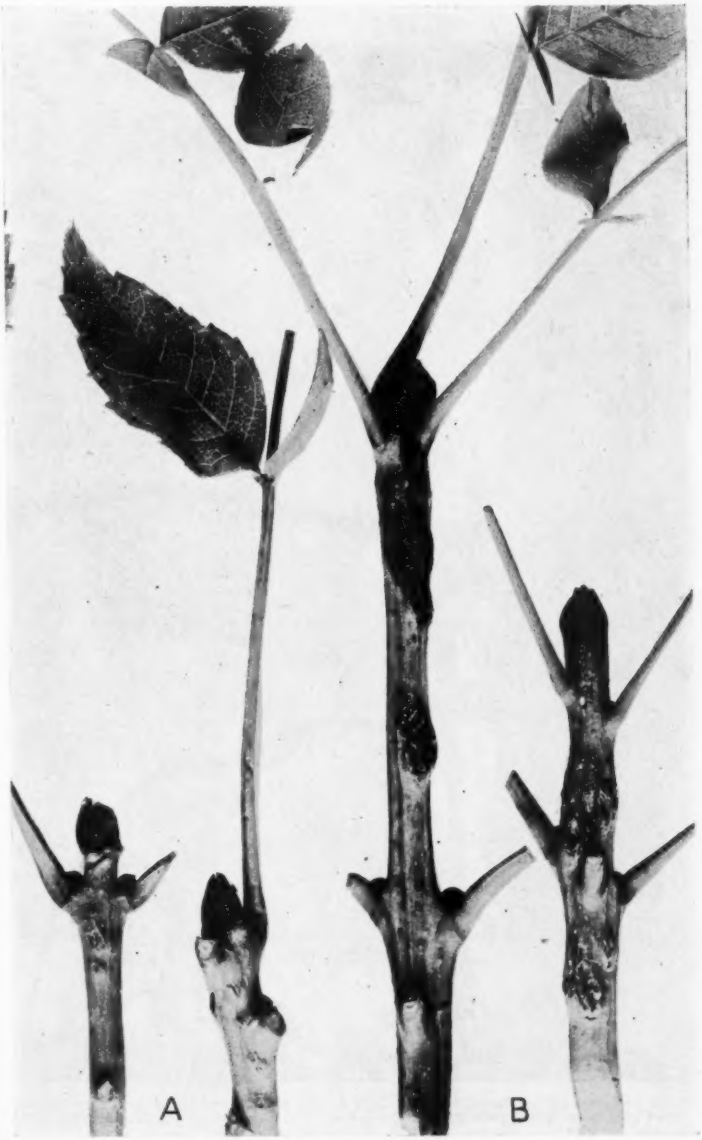

FIGURE 6.—European ash stems inoculated with a reisolation colony of the ash-canker organism. A, Controls punctured with a sterile needle; B, stems inoculated with reisolation colony July 13, 1914; photographed August 10, 1914. All natural size

FIGURE 7.—European ash inoculated July 13, 1914, with organism reisolated from a canker produced on American ash; photographed October 10, 1918. Note cankers forming on small stem. Inoculations were made only on large stem. A little less than natural size

TABLE 1.—*Comparison of the olive-tubercle (Portofino strain) and the ash-canker organisms by general laboratory tests*

Specification	Ash-canker organism	Olive-tubercle organism (Portofino strain)
Colonies on beef-agar plates	Surface colonies smooth, flat, glistening, white, mostly circular, some with entire, some with erose margins. Margins bluish in transmitted light; colonies translucent white in reflected light. Diameter 3 mm in 3 to 4 days at 23° C.	Same as ash-canker organism, except no erose margins, and bluish margins wider in transmitted light.
Beef-agar slants	Margins faintly undulate; growth rather heavy; translucent white spreading	Same as ash-canker organism, except growth not so heavy and surface a little smoother.
Beef-infusion bouillon (Witte's peptone).	Clouded throughout at second day. No pellicle or rim at 4 days (room temperature 25° to 30° C.). Grows better at 25° than at 30°. 8 to 10 days' growth heavier at surface, becoming an incomplete pellicle.	Same as ash-canker organism.
Peptone water (Witte's) 2 per cent solution.	Heavy growth with pellicle	Do.
Fermi's solution	Fine white growth with pellicle, which breaks up into flocks and filaments	Do.
Uschinsky's solution	Growth white, fine clouding throughout. No color change; incomplete pellicle, which breaks up on handling the tube. A viscid swirl rises from bottom of tube on shaking.	Do.
Cohn's solution	Fine white growth throughout, crystals at surface. Old flask cultures show a slight green tinge in the medium.	Same as ash-canker organism, except old flask cultures show no green color.
Bouillon over chloroform	Growth fair but retarded	Same as ash-canker organism.
Toleration of NaCl in beef bouillon	Good growth on bouillon containing 0.5 per cent; no growth in that containing 1, 1.5, or 2 per cent.	Do.
Beef-gelatin plates	Beef-infusion gelatin at 14° C. shows no liquefaction of colonies in 34 days. Then flat, circular, translucent colonies develop with undulating margins. Rugose. Diameter 2 to 3 mm in 6 days; 4 mm in 11 days.	No liquefaction. Margins undulating. Fine internal striae present. Colonies 1.5 to 2.5 mm in diameter at end of 4 days; white, circular, flat, thin.
Beef-gelatin stabs	Slight surface growth at 14° to 16° C. in 5 days; flat, thin, translucent. Faint growth down stab in 27 days. No liquefaction.	Same as ash-canker organism, except white rather than translucent.
Starch jelly	Cream-colored growth in 4 to 9 days. Surface growth yellowish in 31 days; medium pearl-gray at bottom of tube.	Growth white instead of cream-colored in 4 to 9 days. In 31 days surface growth and medium the same as for ash-canker organism.
Litmus milk	Begins to blue in 4 days; gradually becomes dark blue. No coagulation	Same as ash-canker organism, except color change a little slower.
Sterile milk	No change noticed until 20 days, when the milk becomes tan color, deeper than control. No coagulation. Does not form acids in milk; does not coagulate by a lab ferment.	Do.
Gas formation and behavior in fermentation tubes containing 2 per cent of Witte's peptone and 1 per cent of carbon compound as follows:	No gas produced in any of the carbon compounds	Same as ash-canker organism.
Mannite	Heavy growth in open arm of tube; medium dark brown; no rim; no pellicle; neutral litmus paper unchanged.	Same as ash-canker organism, except medium not so dark and small clumps of pseudozoogloeae on the surface.
Glycerin	Heavy growth in open arm and heavy precipitate; rim; medium darkened; neutral litmus paper unchanged.	Same as ash-canker organism.
Cane sugar	Medium, dark brown; heavy growth and a heavy precipitate; rim. Growth continued two-thirds of the way around U of the tube. Faint acid reaction to neutral litmus paper.	Same as ash-canker organism, except medium light brown and clumps of pseudozoogloeae on surface.

TABLE 1.—*Comparison of the olive-tubercle (Portofino strain) and the ash-canker organisms by general laboratory tests*—Continued

Specification	Ash-canker organism	Olive-tubercle organism (Portofino strain)
Gas formation and behavior in fermentation tubes etc.—Continued.		
Galactose	Heavy growth in open arm; heavy precipitate and rim. Clumps of pseudozoogloeae at surface. Color of medium unchanged. Acid reaction to neutral litmus paper.	Same as ash-canker organism.
Dextrose	Heavy growth with clumps of pseudozoogloeae at surface; heavy precipitate; medium darkened slightly. Acid reaction to neutral and blue litmus paper. Liquid in open arm tested pH 6.0.	Same as ash-canker organism, except liquid in open arm tested pH 6.6.
Lactose	Heavy white precipitate in wormlike strand; white rim. Medium darkened. Neutral litmus paper unchanged.	Same as ash-canker organism.
2 per cent peptone water only	Good growth; moderate amount of precipitate; faint rim. Medium darkened slightly. Neutral litmus paper unchanged.	Do.
Litmus-peptone[a] agar	Slight growth in 10 days. No color change in 4 weeks	Do.
Litmus-peptone-dextrose agar	No color change in 10 days	Do.
	In 19 days medium reddened	Do.
	In 29 days no further change	Do.
	In 67 days still only a moderate growth, and red color dulled	At 67 days medium still a good red color.
Litmus-peptone-galactose agar	In 10 days medium of one culture partly red; others unchanged	In 10 days medium of all cultures red.
	In 19 days medium of all cultures red	In 67 days medium still red.
	In 67 days medium of 2 cultures blue and of 1 purple. Moderate growth of organism.	
Litmus-peptone-saccharose agar	Good growth readily	Good growth readily.
	In 10 days medium of all cultures a faint purple	In 10 days medium of all cultures distinct purple.
	In 19 days medium part red and part purple	In 19 days medium purple—no red.
	In 29 days the medium of the various cultures varied from red to purple	In 29 days medium purple—no red.
	In 67 days medium varied from dull red to dull purple	In 69 days medium all dull purple.
Litmus-peptone-glycerin agar	Good growth of organism in 10 days and no color change	No color change in 10 days; good growth.
	In 19 days no color change	In 19 days medium a faint purple.
	In 29 days a trace of blue in medium	In 29 days medium a muddy purple.
	In 67 days no further change	In 67 days no further change.
Litmus-peptone-lactose agar	Moderate growth of organism in 10 days	Same as ash-canker organism.
	In 19 days medium unchanged	Do.
	In 29 days medium still unchanged	Do.
	In 67 days medium blued a little	Do.
Litmus-peptone-maltose agar	Moderate growth in 10 days and no color change	Do.
	In 19 days medium had blued slightly	Do.
	In 67 days no further change	Do.
Litmus-peptone-mannite agar	Good growth and no color change in 10 days	Good growth and medium red in 10 days.
	In 19 days medium had changed to a dull purple	In 19 and 29 days medium still red.
	In 29 and 67 days medium still a dull purple	In 67 days medium a dull purple.

Toleration of acids in neutral beef bouillon containing:		
Malic acid	No growth in medium containing 0.5 per cent or 0.2 per cent, pH 4.9	Same as ash-canker organism.
Oxalic acid	Good growth in medium containing 0.1 per cent, pH 6.2. No growth in medium containing 0.2 per cent, pH 4.7.	Do.
Hydrochloric acid	Good growth in medium containing 0.1 per cent, pH 6.8, and fair growth in medium containing 0.2 per cent, pH 5.6.	Same as ash-canker organism except no growth in medium containing 0.2 per cent hydrochloric acid, pH 5.6.
Production of indol	No indol in 10 days; slight amount in 22 days	No indol in 10 days; slight amount in 21 days.
Hydrolysis of starch	Organism streaked on starch-agar plates and grown for 8 days. Surface of plate flooded with saturated solution of iodine in 50 per cent alcohol. Medium showed destruction of starch extending ½ to 1 cm on each side of growth streak.	Under same treatment as in ash, starch destruction greater. Zone of starch destruction extended 1½ to 2 cm on each side of growth streak.
Production of hydrogen sulphide	None	None.
Nitrate reduction	Tested at 10, 16, and 20 days. No reduction to nitrites	Same as ash-canker organism.
Relation to light as shown by sunlight test.	Tests made at noon in April, August, and October with thinly sown, beef-agar plates on ice bags bottom up, one-half plate exposed to sunlight. Colonies appeared on plates exposed 5 minutes but not on those exposed 10, 20, or 30 minutes. Colonies appeared on covered side of plates. Temperature of ice bag did not exceed 15° to 19° C.	Do.
Temperature relation	Optimum, 23° to 24° C.; maximum, 32°; minimum, 5°. Thermal death point between 43° and 46° when fresh-beef-bouillon cultures were exposed in water bath for 10 minutes.	Optimum, 25° to 26° C.; maximum, 34° to 35°; minimum, 12°. Thermal death point same as in ash-canker organism.
Relation to acid and alkali	In beef-infusion bouillon the pH range is 5.6 to 8.5 with optimum 6.8 to 7.0	In beef-infusion bouillon the pH range is 6.5 to 9+, with optimum 7.0 to 7.2.
Relation to oxygen	Organism an aerobe. Tested in fermentation tubes with various media; also in shake cultures made with beef infusion-peptone agar containing dextrose.	Same as ash-canker organism.
Chromogenesis	On beef agar, white; on beef gelatin, translucent white; on potato cylinders, olive-buff [b] after 7 days' growth.	On beef agar and beef gelatin, white; on potato cylinders, deep olive-buff after 7 days' growth.
Morphology	Short rods; in culture media, single, in pairs, or in short chains; 1 to 4 polar flagella. Rods stained with carbol fuchsin 1.2μ to 3.3μ×0.4μ to 0.8μ (2-day beef-agar culture). No spores.	Rods same as in ash-canker organism, except for a slight difference in size when grown on beef agar 2 days stained the same; 1.2μ to 1.5μ×0.4μ to 0.5μ (2-day beef-agar culture); chains sometimes longer than in ash. Flagella same as in ash-canker organism. No spores.
Staining reactions	Stained readily with carbol fuchsin, methyl violet, and dahlia. Gram-negative and not acid-fast.	Same as ash-canker organism.
Index number	5,620–31,629–1,262	Do.

[a] Witte's peptone was used throughout this test.

[b] The colors mentioned in this paper are according to Ridgway. (Ridgway, R. color standards and color nomenclature. 42 p., illus. Washington, D. C. 1912.)

TABLE 2.—*Comparison of cultural and physiological tests of the olive-tubercle organism (Portofino strain) and of the ash-canker organism with Petri's tests of his olive-tubercle strain*

[Petri's formulas were followed]

Medium	Olive organism (Petri's Italian strain)	Olive organism (Portofino strain)	Ash organism (Vienna strain)
Nutrient agar (Witte's peptone, 1 g; anhydrous dextrose, 1 g; agar-agar, 1.5 g; distilled water, 100 g).	Colonies visible under microscope (×50) after 24 to 48 hours if bacteria came from another culture; after 3 to 6 days if they came from the tubercles or from the olive fly (temperature 17° C.). Maximum diameter, 2.2μ to 5μ. Colony round, white, translucent, then opaque; entire margins.	Colonies up in 48 hours; very small, round, translucent white; entire margins; thicker than ash colonies.	Colonies up in 48 hours; translucent white, but many not round; erose margins. 2 to 3.5 mm in diameter.
	Slant cultures: Extended milk-white growth; margins undulate and a little raised. Not viscid.	Slant cultures: Colonies massed together making an uneven, almost corrugated surface; almost opaque. Thicker and heavier than ash colonies; in 6 days, flattened out and spread somewhat. Not viscid.	Slant cultures: Minute colonies, rather opaque; in 6 days, fused somewhat and surfaces less corrugated. Not viscid.
Nutrient agar (Merck's dry peptone from flesh, 1 g; anhydrous pure dextrose, 1 g; agar-agar, 1.5 g; distilled water, 100 g).	After 60 hours, first colonies noted under microscope; growth soon arrested.	No colonies	No colonies.
	Slant cultures: Poor development, soon ceasing. Inoculations often sterile.	No growth on slant agar	No growth on slant agar.
Nutrient agar (decoction of young branches of olive neutralized, 100 g; cane sugar or dextrose, 1 g; agar, 1.5 g).	Slow development. Colonies small; white. Tannic substances an obstacle to development.	No growth	No growth.
Nutrient gelatin (Witte's peptone, 1 g; dextrose, 1 g; gelatin, 10 g; distilled water, 100 g).	Small white translucent colonies on plates	Test 1: Colonies up in 11 days; small and white; most of them round, a few indented.	Test 1: Colonies up in 6 days, very tiny; in 12 days, largest 1.5 mm in diameter; white and deeply indented at margins.
	Slant culture: Growth similar to that on dextrose-peptone agar.	Test 2: Colonies up in 7 days; under hand lens, some with fringelike growth at margin, others erose, like ash colonies.	Test 2: Colonies up in 3 days, pin point; in 7 days, only 1 mm in diameter; white with darker centers, erose margins; only a few round or nearly round.
	Stab culture: Scanty development in canal; colony at surface round; no liquefaction.	Stab cultures. Test 1: No growth. Test 2: Tiny white opaque colonies on surface at 20 days; feathery development in canal; no liquefaction.	Stab cultures. Test 1: Growth after 6 days. Test 2: Growth after 3 days; on surface, small colonies, white, opaque, irregular margins; midway, small bunch of colonies very fine and clustered; no liquefaction.
Bean agar (broth of beans, neutralized, 100 g; agar, 1.5 g).	Substratum in which degeneration of the bacteria was much retarded from 4 to 6 months. Effect increased by addition of trace of sodium or potassium phosphate.	Colonies grew well, but not so large as ash; granular markings, not seen on surface.	Good medium for this organism. Colonies on plates not always round; erose margins. Twice the size of *Bact. savastanoi* (Portofino).
		Slant cultures; good growth, very like beef agar.	Slant cultures; good growth, very like those on beef agar.

Steamed potato:			
Test 1	Colony viscous, threadlike, pearl white	Same as ash organism	Growth in 3 days, pale olive-buff; viscid; medium slightly grayed.
	Starch transformed to maltose and amylodextrin.		Not tested for maltose and amylodextrin.
	Substratum not browned with age of culture		In 6 days, medium brownish gray; in 25 days, wood-brown.
Test 2		8-day cultures. Mashed up, with water, 2 cc of strong iodine in alcohol gave blue color not so deep as control; no purple.	8-day cultures: Mashed up with water, 2 cc. of strong iodine in alcohol gave blue color not so deep as control; no purple.
		Retained color for about half an hour, then gradually grew lighter.	Color faded entirely in 5 minutes; 1 cc iodine added; color faded out again immediately. 1 cc iodine added; color remained same shade as *Bact. savastanoi* (Portofino) for half an hour, then gradually grew lighter. Control retained blue color.
Steamed carrot	White, pearllike, salient, nonviscid growth	No growth in 3 days. Very faint white growth in 4 days.	Growth thin, white, in 3 days.
		In 6 days, growth still faint; not viscid; medium unchanged.	In 6 days, growth not abundant but heavier than *Bact. savastanoi* (Portofino); not viscid; medium unchanged.
		In 25 days, a heavy white precipitate; growth still thin; medium unchanged.	In 25 days, a heavy white precipitate; growth still thin; medium unchanged.
Bile-salt agar (sodium-glycocholate, 2 g; sodium taurocholate, 2 g; agar, 1.5 g; water, 100 g.)	Good development. Colonies round and white.	Test 1: No growth	Test 1: No growth.
		Test 2: No growth	Test 2: No growth.
Cohn's solution	Clouding after 48 hours at 15° C. At 25° inappreciable hastening of growth. Bacteria united into a rim; elongated; motile; slender. After a month, abundant accumulation of crystals of double phosphate of ammonia and magnesia.	Same as ash-canker organism up to eleventh day.	In 3 days, fair amount of growth.
		In 60 days, growth was not so heavy as ash organism; but there were more crystals.	In 5 days, thin pellicle with crystals; fine white growth throughout.
		In 81 days, no color change. Bacteria motile; elongated forms abundant; some chains.	In 11 days, no difference in ash-canker organism or in *Bact. savastanoi* (Portofino); many crystals floating on surface; fine white growth throughout.
			In 60 days, rather heavy clouding, but not many crystals.
			In 81 days, faint tinge of green. Crystals still not abundant. Bacteria motile; a few elongated forms; no chains.
Cohn's solution, 100 g; anhydrous dextrose, 1 g.	Growth very rapid and abundant; thick pellicle consisting of rods united into bunches, many motile; numerous crystals (1–1.5 mm long) on pellicle and along walls of tube.	In 5 days coarse flocks; no pellicle; no crystals.	In 5 days thin pellicle; coarse flocks; no crystals.
	In about 20 days superficial strata of liquid, color of green peas but lighter.	In 11 days no rim; faint almost imperceptible pellicle made up of crystals.	In 11 days rim; incomplete pellicle; crystals on surface.
		In 21 days no rim; no pellicle except floating crystals about 1 mm in diameter. Bacterial growth not heavy; rods united into bunches; precipitate.	In 21 days pellicle still thin; rim present; many crystals at surface, largest not more than 1 mm in diameter.
	Abundant white precipitate in bottom of tube		Bacterial growth in pellicle made up of rods united into bunches; precipitate; no green color in medium.
		In 28 days no green color	In 28 days one ash culture had a faint trace of green.
		In 80 days still no green color	In 80 days decided green-pea color in all 3 cultures.

TABLE 2.—*Comparison of cultural and physiological tests of the olive-tubercle organism (Portofino strain) and of the ash-canker organism with Petri's tests of his olive-tubercle strain*—Continued

Medium	Olive organism (Petri's Italian strain)	Olive organism (Portofino strain)	Ash organism (Vienna strain)
Witte's peptone, 1 g; anhydrous dextrose, 1 g; glycerin, 2 g; water, 100 g.	Rapid clouding. Motile and elongated forms. Interrupted pellicle consisting of a hyaline mucous substance apparently from degeneration of bacterial membranes.	Mucous pellicle in 4 days; uniform growth below. Pellicle under microscope has motile but no elongated forms. Definite threads (protoplasmic) sometimes connect one bacterium to another; threads sometimes free from bacteria; ends of bacteria sometimes drawn out nearly to a point by thread. Degenerate forms also in pellicle mass. Some forms rounded a little at extremities	No pellicle; clouding not so heavy as in *Bact. savastanoi* (Portofino). Motile but no elongated forms. No gelatinous threads. Some forms rounded a little at extremities.
Merck's peptone from flesh, 1 g; anhydrous dextrose, 1 g; glycerin, 2 g; water, 100 g.	In 3 days, at 25° C., liquid still clear In 4 days almost inappreciable growth visible to naked eye. In succeeding days, clouding still very slight	No growth	No growth.
Neutral beef bouillon with Witte's peptone.	Growth not abundant; thin white pellicle	Same as ash organism	Growth faint up to eighth day; then heavier; abundant by sixteenth day. No rim or pellicle.
Uschinsky's solution	Moderate development, less than in Cohn's solution; bacteria motile and elongated.	Faint growth in 3 days, a trifle more than in ash organism. In 11 days no rim or pellicle. In 19 days growth still slight. Bacteria motile and elongated 4 to 10 times usual length. In 21 days same as ash organism In 48 days no further change	Faint growth in 3 days. In 11 days no rim or pellicle. In 19 days growth still slight. Bacteria motile and elongated 4 to 10 times usual length. In 21 days growth still faint and less than in Cohn's solution. In 48 days no further change.
Sterile milk	Good development. Milk not coagulated. After 20 days, reaction neutral.	Same as ash organism	No change noted until seventeenth day, then indication of clearing. In 25 days milk entirely cleared without coagulation. In 30 days, milk pale olive buff. Reaction slightly alkaline to litmus, nearly neutral to phenolphthalein.
Test for reduction of nitrates (Witte's peptone, 1g; nitrate of potash, 0.3 g; distilled water neutralized with sodium hydrate, 100 g).	No reduction of nitrates	No reduction of nitrates	No reduction of nitrates.

Whey of milk with litmus. (The curd was precipitated with dilute hydrochloric acid, then the whey was neutralized by adding sodium hydroxide; litmus added gave a pale lavender color.)	No acid produced	Same as ash organism, except at 3 days the color of medium was a little deeper blue. In 8 days medium forget-me-not blue. No acid produced.	Good growth in 3 days; medium slightly blued. Not so deep a color as *Bact. savastanoi* (Portofino). In 8 days medium forget-me-not blue. No acid produced.
Witte's peptone, 1 g; sodium chloride, 0.5 g; distilled water, 100 g; rosolic acid (0.5 per cent solution), 1 g.	In 3 days color changed to yellowish (production of acid). In 15 days return of reddish color (production of alkali).	Good growth. Slight change in color of medium in 3 days; paler than control. In 9 days medium several shades lighter than control but never yellowish. In 2 months medium darker than control; same color as ash organism. Tested with litmus paper when cultures were 3 months old. All gave an alkaline reaction. Controls faintly acid.	Good growth; color never became lighter. In 2 months medium several shades darker than controls, same as *Bact. savastanoi* (Portofino). Tested with litmus paper when cultures were 3 months old. All gave an alkaline reaction. Controls faintly acid.
Carrot sterilized at a low temperature to test for production of cytase.	The prolonged development did not cause disintegration of cells; apparently no production of cytase. Juice from such cultures was inactive on middle lamellae of cell walls of sections of fresh carrot.	Same as ash organism. Cytase not developed.	64-day-old cultures mashed and juice extracted. Raw carrots washed carefully, then immersed in $HgCl_2$ (1:1,000) for 5 minutes and rinsed off in sterile water. Slices placed in deep Petri dishes and juice poured on the cut surfaces. No change in 10 days.
Cohn's solution, 100 g; potato starch, 0.5 g.	After 2 or 3 days starch transformed to maltose and amylodextrin. No active amylase extracted from the cultural liquid.	Same as ash organism up to 5th day, then showed more and larger crystals.	In 3 days, white growth near surface of liquid. In 5 days, thin pellicle and fine white growth throughout. Crystals in pellicle and on surface of starch layer at bottom of tube.
Witte's peptone, 1 g.; saccharose, 2 g; water, 100 g.	Sample of cultural fluid tested with Fehling's fluid. After 8 days' growth another sample of the organism showed a certain quantity of glucose. The cultural fluid passed through a Kitasato filter, with thymol and 0.5 per cent saccharose added, showed presence of invertase.	Same as ash organism.	After 8 days' growth, culture tested with Fehling's solution and glucose found. Greater reduction in ash organism than in *Bact. savastanoi* (Portofino). Some cultures killed by heating at 55° C. for 20 minutes. Allowed to stand for several days, then transfers made to see if cultures were alive. No growth in transfers in 3 days, so heated cultures were considered dead. A crystal of thymol was added to each culture and 0.5 per cent saccharose. No growth in 3 days, when test showed the presence of invertase.
Dunham's solution for production of indol.	A culture 20 days old showed a reddish color. Production of indol not very abundant.	21-day-old cultures tested with sulphuric acid and sodium nitrite (fresh solution) gave a pink color without heating. Deep vinaceous.	21-day-old cultures tested for indol with sulphuric acid, sodium nitrite (fresh solution) gave a pink color after heating, but not so deep as *Bact. savastanoi* (Portofino). Vinaceous.

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TABLE 2.—*Comparison of cultural and physiological tests of the olive-tubercle organism (Portofino strain) and of the ash-canker organism with Petri's tests of his olive-tubercle strain*—Continued

Medium	Olive organism (Petri's Italian strain)	Olive organism (Portofino strain)	Ash organism (Vienna strain)
Uschinsky's solution, 100 g; xylose, 3 g. For production of gas.	Cultures in fermentation tubes: Slight production of gas; fluid became acid.	No gas production	No gas production.
Witte's peptone, 1 g; indigo carmine (0.5 per cent solution), 2 g; distilled water, 100 g:			
Test 1	Bottom of tube discolored after 48 hours; after 20 days colored ring remained at the surface.	Moderate growth. Color changed uniformly to a darker shade of blue-green, darker than the ash organism.	Moderate growth. No discoloring in rings. Medium deeper blue-green.
Test 2	Color restored, but less intense, by current of air passed into fluid.	Moderate amount of growth in 4 days. Color unchanged. Controls faded. In 6 to 21 days, color dark cinnabar green. Controls faded out entirely.	Same as *Bact. savastanoi* (Portofino) except (at 6 to 21 days) color a tint deeper with a trifle more blue but still dark cinnabar green.
Phosphate of potash, 0.02 g; agar, 1.5 g; mannite, 2 g; distilled water, 100 g.	Rapid development, gradually slowing down; colony small. Bacteria very motile. Some motionless, united in chains, parallel to each other in sinuous bundles. After 15 days vacuolated individuals were observed (degenerated forms).	Pin-point colonies up in 4 days. In 6 days pearllike, translucent, white, higher in center, 1 to 1.5 mm in diameter. Fish-scale-like internal markings. In 8 days colonies 2.5 mm in diameter; raised center indented. In 10 days bacteria motile; not in chains; no vacuolated forms seen.	Like *Bact. savastanoi* (Portofino) until 6th day. In 10 days largest colonies 1.5 mm in diameter; not so thick in center as *Bact. savastanoi* (Portofino). Bacteria motile; not in chains; no vacuolated forms seen.
Winogradski's solution, 100 g; with:			
Dextrose, 3 g	Moderate growth. Development stopped after 15 days. No butyric fermentation. Bacteria transferred to other substrata multiply actively.	Test 1: No growth Test 2: Mere trace of growth even after 4 weeks	Test 1: No growth. Test 2: Clouding in 3 days; heavy growth and pellicle in 2 weeks.
Calcium proprionate, 0.5 per cent.	Less development than in Winogradski's solution with dextrose.	Test 1: No growth Test 2: No growth	Test 1: No growth. Test 2: No growth.
Mannite, 3 per cent	Scarcely appreciable development	Test 1: No growth Test 2: No growth in 6 days; trace in 9 days; same at 4 weeks.	Test 1: No growth. Test 2: No growth in 6 days; a fair amount of clouding with light pellicle in 9 days; very little heavier in 4 weeks.
Saccharose, 3 per cent	do	Test 1: No growth Test 2: No growth in 5 days; mere trace in 12 days; no heavier in 4 weeks.	Test 1: Faint growth in all cultures. Test 2: Faint white growth in 5 days; same in 12 days; no further change in 4 weeks.
Arabinose, 3 per cent	Good development, equaling that in Winogradski's solution with dextrose.	Test 1: No growth Test 2: No growth	Test 1: No growth. Test 2: Slight growth in 4 weeks.

Xylose, 3 per cent	....do	Test 1: No growth Test 2: No growth	Test 1: No growth. Test 2: No growth in 6 days; faint growth in 12 days; still faint in 4 weeks.
Lactose, 3 per cent	Scarcely appreciable development	Test 1: No growth Test 2: No growth	Test 1: No growth. Test 2: Trace of growth in 5 days; no heavier in 4 weeks.
Merck's peptone from flesh, 1 g; anhydrous dextrose, 1 g; glycerin, 2 g; water 100 g; tartaric acid, 0.07 per cent.	No development	No development	No development.
Oils:			
Otto Rahn's solution (phosphate of potash, 0.5 g; sulphate of magnesium, 0.1 g; phosphate of ammonia, 0.5 g; ferric chloride, trace; hydrochloric acid, trace; distilled water, 100 g) plus 2 per cent olive oil.	In 20 to 30 days increase of free fatty acids minimum, showing extremely weak lipolytic action. Drops of oil in the cultures, consisting of a very delicate external wrinkled pellicle within which were seen vacuoles, more or less large, filled with bacteria.	Faint clouding in 4 days. At 18 days, still a faint clouding; oil splitting up into globules; slight lipolytic action.	Same as *Bact. savastanoi* (Portofino).
Cohn's solution plus 2 per cent olive oil.	....do	Good growth in 4 days; white layer at surface; flocks throughout. In 13 days crystals hung from underside of pellicle. In 18 days growth still heaviest at top. No splitting of oil.	Do. Do. Do.
Peptone water, precipitated carbonate of lime, and 2 per cent olive oil.	....do	Growth in 4 days. No splitting of olive oil even at 21 days.	Do.
Resistance to copper, lithium, or nickel in Cohn's solution plus dextrose and:			
Copper sulphate— 1:500,000	Growth occurred	Medium clouded in 3 days. Many crystals at surface in 9 days.	Do.
1:200,000	No growth	No growth	No growth.
1:100,000	....do	....do	Do.
Nickel sulphate 1:10,000	Growth occurred	No growth in 2 days; fair amount of growth in 5 days.	No growth in 8 days; growth in 18 days, partial rim, no pellicle.
Lithium sulphate 1:1,000.	....do	Slight growth in 3 days; good growth in 9 days.	Slight growth in 3 days; good growth in 9 days.

TABLE 2.—*Comparison of cultural and physiological tests of the olive-tubercle organism (Portofino strain) and of the osh-canker organism with Petri's tests of his olive-tubercle strain*—Continued

Medium	Olive organism (Petri's Italian strain)	Olive organism (Portofino strain)	Ash organism (Vienna strain)
Resistance to the following percentages of tartaric acid in: Witte's peptone, 1 g; anhydrous dextrose, 1 g; glycerin, 2 g; water, 100 g—			
0.1 per cent	No growth	No growth	No growth.
0.07 per cent	Weak growth	do	Do.
0.03 per cent	Elongation of bacteria	Good growth; no pellicle	Heavy growth; thin white pellicle.
		In 6 days, many cross (x) forms. A few elongated forms and a few chains, but bacteria were mostly short.	In 6 days, all short forms, no elongated ones nor x forms. Pellicle composed largely of dead bacteria.
		In 15 days, short forms prevalent, clumps present and a few Y's; bacteria motile.	In 15 days, short forms prevalent, clumps present and a few Y's; bacteria motile.
0.005 per cent	do	Good growth; no pellicle	Good growth; incomplete pellicle and rim.
		In 6 days short chains, some elongated forms and clumps.	In 6 days, no clumps of bacteria nor elongated forms.
		In 15 days small clumps, short chains, and a few Y's; bacteria motile.	In 15 days small clumps, short chains, and a few Y's; bacteria motile.

The detailed cultural work outlined in Tables 1 and 2 was carried out in order to ascertain whether the differences between the ash-canker and the olive-tubercle organisms would justify the separation of the ash organism as a distinct species.

Although the olive-tubercle and the ash-canker organisms did not cross-inoculate in any of the tests and differed in some of their cultural characters, they were alike in the majority of the tests; morphologically they are essentially alike. The difference may be accounted for by the influence of the host on its particular parasite, an influence which has persisted to a marked degree during the 17 years in which the two organisms have been subcultured. The ash-canker organism, therefore, has not been made a separate species, but is here regarded as a variety of the olive-tubercle organism, *Bacterium savastanoi*, for which the varietal name *fraxini* is suggested.

The differences between Petri's olive-tubercle organism and the one used for this work (Portofino isolation) as indicated in Tables 1 and 2 are probably due to the differences in strains of the same organism. It may be noted that Petri's results correspond sometimes to those obtained with the olive-tubercle (Portofino) organism, sometimes to those obtained with the ash-canker organism, and sometimes to neither. Occasionally it happens that all three organisms give similar results. It is known that various bacteria exist in a variety of strains, and undoubtedly the olive-tubercle organism is no exception.

THE NEW VARIETY AND THE TYPE SPECIES COMPARED

Bacterium savastanoi fraxini, n. var.

The variety *fraxini* differs from *Bacterium savastanoi* mainly in specific host reactions, for the two organisms are only slightly separable by cultural or morphological characters. Table 3 shows the important differences between the two organisms.

TABLE 3.—*Comparison of Bacterium savastanoi fraxini, n. var., with Bact. savastanoi*

Item	Bact. savastanoi fraxini	Bact. savastanoi
Pathogenicity	Produces cankers on ash trees; is not infectious to olive trees.	Produces tubercles on olive trees; is not infectious to ash trees.
Temperature range	5° to 32° C.	12° to 35° C.
pH range	5.6 to 8.5.	6.5 to 9+.
Cohn's solution	A green color develops in old flask cultures.	No green color in old flask cultures.
Size of organisms from cultures of same age and stained the same.	1.2μ to 3.3μ×0.4μ to 0.8μ.	1.2μ to 1.5μ×0.4μ to 0.5μ.

TREATMENT OF THE DISEASE

The organism causing canker on ash, like the olive-tubercle organism, is a wound parasite, and the treatment recommended for olive trees affected with tubercles may be suggested for the ash trees affected with cankers, namely, skillful pruning. As is usually recommended in treatment of this kind, the cut surfaces should be disinfected, as well as the pruning knives each time they are used.

Possibly a germicidal spray or germicidal paint might be used effectively at the earliest stage of the disease, when the split in the bark is very small and fairly regular; but in the later stage, when the

bark has become corrugated and corky, such treatment would result only in destroying the surface bacteria.

Since a slime containing bacteria oozes from the cankers when they are moist, it is quite possible that the disease may be spread from one branch to another by rain, the organism entering the tissues through very small wounds.

SUMMARY

A bacterial organism isolated from a canker disease of the European ash tree is likewise infectious to the American ash, causing the same type of lesion. The disease has not been reported on the American ash in this country. The cankers of the European ash vary in size from small cracks with thickened margins to irregular fluted outgrowths several inches in length and width, with cavities extending into the wood. They increase in size and number from year to year on both trunk and branches.

The organism isolated from the ash cankers is similar to *Bacterium savastanoi* E. F. Smith, which produces tubercles on olive trees in California, Italy, and other countries. Although the organisms do not cross-inoculate—the ash proving noninfectious to the olive and the olive noninfectious to the ash—both cultural and morphological tests show that they are essentially alike. The ash-canker organism is therefore regarded as a variety of the olive-tubercle organism, *Bact. savastanoi*, and the name *Bact. savastanoi* variety *fraxini* is suggested.

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NITROGEN-BALANCE STUDIES WITH VARIOUS FISH MEALS[1]

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INTRODUCTION

In an earlier report[2] of fish-meal studies by the agricultural experiment station at Cornell University, it was shown that, as measured by growth experiments with rats, the protein of a vacuum-dried white fish meal was superior to that of a steam-dried menhaden meal. Both products proved superior to a flame-dried menhaden meal. It seemed probable that the differences in heat treatment were responsible, at least in part, for the differences in protein efficiency. It was recognized that the effect of higher temperature might show itself either in a lower digestibility or in a lower utilization of the absorbed nitrogen. It was therefore deemed desirable to extend the previous studies by the use of methods which would give more specific information regarding the differences in protein efficiency. The present paper reports the results of nitrogen-balance studies in which the three products previously mentioned were fed to rats and pigs.

The vacuum-dried white fish meal was a commercial product and consisted of the heads, tails, fins, and adhering flesh obtained as a by-product of the fillet industry and dried in vacuum under 105° F. It did not contain the entrails. The flame-dried menhaden meal was also a commercial product containing the whole fish, less most of the oil. This product was dried by direct heat at temperatures of approximately 500° F. The steam-dried menhaden meal was a product experimentally produced by the Bureau of Fisheries.[3] As compared with the flame-dried meal, it was undoubtedly more carefully handled throughout the manufacturing process as well as dried at a much lower temperature. The analyses of the samples used in the present study are shown in Table 1. Two experiments with growing rats and one with growing pigs were carried out, in which Mitchell's method[4] of computing the biological values from the nitrogen-balance data was used.

TABLE 1.—*Percentage analyses of the fish meals studied*

Fish meal	Moisture	Ash	Crude protein	Ether extract
Flame-dried menhaden	5.12	21.13	57.95	6.13
Steam-dried menhaden	7.00	16.50	59.68	11.83
Vacuum-dried white	9.90	19.25	62.02	2.63

[1] Received for publication Dec. 4, 1931; issued May, 1932. This paper presents a part of a thesis presented to the graduate school of Cornell University in partial fulfillment of the requirements for the degree of doctor of philosophy.

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RAT EXPERIMENTS

In each experiment six rats were used. In the first, three females were included, but in the second, males only were employed. After from five to seven days on a nitrogen-free ration consisting of 15 per cent of butterfat, 12 per cent of lard, 2 per cent of cod-liver oil, 4 per cent of salt mixture (Osborne and Mendel), and 67 per cent of starch, the urine and feces were collected for a 7-day period. Then followed three 10-day experimental periods in which the three fish meals were fed at such levels that their proteins formed 10 per cent of the ration, the calorific and mineral content being kept the same. Vitamin B concentrate (Osborne and Wakeman) was included in the ration in the first experiment at a 0.15 per cent level and in the second experiment at a 1.6 per cent level.[5] The collections of excreta were made the last seven days of each 10-day period. The experiments were each ended as they were begun, with a nitrogen-free period. The diets and all the excreta were analyzed for total nitrogen by the Kjeldahl method.

The data obtained in the first and second rat experiments are summarized in Tables 2 and 3 respectively. The two values, the digestion coefficient and the biological value, express as percentages the part of the food nitrogen which is realized by the body at each of the two points in the organism where waste occurs. The digestion coefficient represents the percentage of crude protein ($N \times 6.25$) digested and thus saved for body use. The biological value expresses the percentage of absorbed protein which has been used in anabolism.

By averaging all the protein-digestion coefficients obtained for a given product, as listed in Tables 2 and 3, the following mean values are obtained: Flame-dried menhaden meal, 62.2 ± 1.15; steam-dried mendaden meal, 73.2 ± 0.96; vacuum-dried white meal, 80.7 ± 0.73. Thus, the vacuum-dried white fish meal showed an advantage of 7.5 ± 1.20 over steam-dried menhaden, the difference being 6.2 times its probable error. The digestibility of the steam-dried menhaden showed an advantage of 11.0 ± 1.50 over the flame-dried menhaden fish meal, the difference being 7.3 times its probable error.

By averaging the biological values in Tables 2 and 3 the following mean values are obtained: Flame-dried mendaden meal, 71.7 ± 1.54; steam-dried menhaden meal, 77.4 ± 3.15; vacuum-dried white meal, 83.4 ± 1.83. Analysis of these averages of values from both Table 2 and Table 3 reveals the fact that the difference of 5.7 ± 3.51 between the biological values of flame-dried menhaden and steam-dried menhaden is not significant. The same is true of the difference of 6.0 ± 3.64 between steam-dried menhaden and vacuum-dehydrated white fish meal. The large probable errors which keep these differences from being considered significant are due primarily to the high degree of variation shown by certain of the values obtained in the first experiment, which will be discussed later. The difference of 11.7 ± 2.39 between flame-dried menhaden and vacuum-dried white fish meal is clearly significant, the difference being 4.9 times its probable error. The white fish meal is shown to be distinctly more efficiently used in anabolism than is the flame-dried menhaden. This is borne out by the swine experiment, which is discussed later.

[5] In the first experiment, a minimum amount of vitamin B concentrate was used to avoid too great an addition of nitrogen from a source other than fish meal. The larger amount used in the second experiment was based upon a test of the potency of the product used.

TABLE 2.—*Data obtained with male and female rats in first experiment to determine the protein efficiency of fish meals*

Rat	Initial weight	Final weight	Average daily food intake	Average daily N intake	Average daily fecal N	Estimated daily metabolic N	Average daily food N in the feces	Average daily absorbed N	Average daily urinary N	Estimated daily endogenous N	Average daily food N in the urine	Average daily food N utilized	Digestion coefficient	Biological value
	Grams	*Grams*	*Grams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Per cent*	*Per cent*
NITROGEN-FREE DIET														
Number 1♂	108	99	4.61	.....	12.4	[a] 2.69	.....	.....	32.1	[b] 31.0	.....	.....	.....	.....
Number 2♀	130	125	6.78	.....	17.4	[a] 2.57	.....	.....	21.3	[b] 16.7	.....	.....	.....	.....
Number 3♂	88	79	2.80	.....	6.9	[a] 2.46	.....	.....	61.4	[b] [c] 25.1	.....	.....	.....	.....
Number 4♀	129	119	6.86	.....	17.7	[a] 2.58	.....	.....	25.6	[b] 20.6	.....	.....	.....	.....
Number 5♂	102	91	3.77	.....	11.5	[a] 3.05	.....	.....	26.7	[b] 27.7	.....	.....	.....	.....
Number 6♀	116	115	6.24	.....	12.5	[a] 2.00	.....	.....	34.0	[b] 29.4	.....	.....	.....	.....
FLAME-DRIED MENHADEN FISH MEAL DIET														
Number 1♂	101	101	4.80	76.2	33.6	12.6	21.0	55.2	38.2	28.5	9.7	45.5	56	82
Number 2♀	132	129	4.92	78.1	36.3	12.7	23.6	54.5	38.6	22.3	16.3	38.2	54	70
Number 3♂	98	104	5.51	87.5	36.2	14.2	22.0	65.5	34.0	24.0	10.0	55.5	59	85
Number 4♀	137	129	4.13	65.6	30.4	10.3	20.1	45.5	47.9	27.7	20.2	25.3	54	56
Number 5♂	125	127	5.73	91.0	33.2	15.2	18.0	73.0	46.4	24.1	22.3	50.7	64	69
Number 6♀	131	131	5.53	87.8	34.6	12.2	22.4	65.4	53.1	28.0	25.1	40.3	61	62
STEAM-DRIED MENHADEN FISH MEAL DIET														
Number 1♂	113	112	4.56	70.0	21.2	11.7	9.5	60.5	35.7	28.6	7.1	53.4	70	84
Number 2♀	130	129	4.83	74.1	23.6	12.7	10.9	63.2	38.5	22.8	15.7	47.5	68	75
Number 3♂	109	109	5.07	77.8	26.6	13.4	13.2	64.2	46.3	25.2	21.1	43.5	66	67
Number 4♀	130	121	4.41	67.7	20.8	10.9	9.9	57.8	65.4	26.2	39.2	18.6	69	32
Number 5♂	106	100	5.03	77.2	24.3	14.7	9.6	67.6	30.7	24.8	5.9	61.7	69	91
Number 6♀	117	120	4.91	75.4	20.6	10.2	10.4	65.0	34.8	31.6	3.2	61.8	73	95
VACUUM-DRIED WHITE FISH MEAL DIET														
Number 1♂	127	138	6.84	112.8	23.8	17.2	6.6	106.2	42.9	30.1	12.8	93.4	79	88
Number 2♀	135	131	4.66	76.7	19.2	12.3	6.9	69.8	55.6	23.9	31.7	38.1	75	55
Number 3♂	97	102	4.53	74.5	16.0	11.4	4.6	69.9	33.6	24.3	9.3	60.6	79	87
Number 4♀	126	139	6.04	99.4	19.8	15.3	4.5	94.9	39.2	27.4	11.8	83.1	80	88
Number 5♂	117	123	6.44	105.9	27.2	17.9	9.3	96.6	37.4	26.4	11.0	85.6	74	89
Number 6♀	117	134	6.03	99.2	21.0	12.9	8.1	91.1	42.7	30.1	12.6	78.5	79	86

[a] Per gram of food. [b] Per 100 grams live weight. [c] This value was estimated as not enough food was eaten during this period to supply the energy requirements of the rat.

TABLE 2.—*Data obtained with male and female rats in first experiment to determine the protein efficiency of fish meals*—Continued

NITROGEN-FREE DIET

Rat	Initial weight	Final weight	Average daily food intake	Average daily N intake	Average daily fecal N	Estimated daily metabolic N	Average daily food N in the feces	Average daily absorbed N	Average daily urinary N	Estimated daily endogenous N	Average daily food N in the urine	Average daily food N utilized	Digestion coefficient	Biological value
	Grams	*Grams*	*Grams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Per cent*	*Per cent*
Number 1♂	135	125	5.74		14.1	[a] 2.46			25.9	[b] 19.9				
Number 2♀	129	125	5.66		15.1	[a] 2.67			23.5	[b] 18.5				
Number 3♂	106	99	4.13		11.2	[a] 2.71			23.1	[b] 22.5				
Number 4♀	124	114	6.43		15.6	[a] 2.43			25.0	[b] 21.0				
Number 5♂	127	120	5.59		14.1	[a] 2.52			20.1	[b] 16.3				
Number 6♀	131	123	5.36		12.2	[a] 2.28			23.8	[b] 18.7				

TABLE 3.—*Data obtained with male rats in second experiment to determine the protein efficiency of fish meals*

NITROGEN-FREE DIET

Rat	Initial weight	Final weight	Average daily food intake	Average daily N intake	Average daily fecal N	Estimated daily metabolic N	Average daily food N in the feces	Average daily absorbed N	Average daily urinary N	Estimated daily endogenous N	Average daily food N in the urine	Average daily food N utilized	Digestion coefficient	Biological value
	Grams	*Grams*	*Grams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Per cent*	*Per cent*
Number 1	88	74	4.12		7.7	[a] 1.87			22.3	[b] 27.5				
Number 2	92	75	4.24		8.2	[a] 1.93			24.6	[b] 29.5				
Number 3	91	77	3.38		6.8	[a] 2.01			27.2	[b] 32.4				
Number 4	107	91	4.54		8.7	[a] 1.92			25.2	[b] 25.5				
Number 5	108	90	3.88		7.3	[a] 1.88			25.9	[b] 26.2				
Number 6	95	77	4.27		7.3	[a] 1.71			27.1	[b] 31.5				

FLAME-DRIED MENHADEN FISH MEAL DIET

Number 1	70	84	5.28	87.9	24.9	10.3	14.6	73.3	38.2	20.6	17.6	55.7	72	76
Number 2	85	92	6.01	100.0	35.7	12.7	23.0	77.0	46.5	24.8	21.7	55.3	64	72
Number 3	122	126	6.40	106.5	44.3	14.9	29.4	77.1	53.2	29.0	24.2	52.9	58	69
Number 4	161	165	8.06	134.1	45.9	16.0	29.9	104.2	69.4	37.0	32.4	71.8	66	69
Number 5	112	119	5.06	84.2	27.1	9.3	17.8	66.4	43.6	26.9	16.7	49.7	68	75
Number 6	122	140	9.64	160.4	48.1	20.5	27.6	132.8	71.5	37.7	33.8	99.0	70	75

STEAM-DRIED MENHADEN FISH MEAL DIET

Number 1	116	129	7.84	138.6	28.3	16.9	11.4	127.2	54.8	31.0	23.8	103.4	80	81
Number 2	123	134	7.93	140.2	30.1	19.5	10.6	129.6	61.1	32.3	28.8	100.8	79	78
Number 3	112	122	6.36	112.4	32.8	14.1	18.7	93.7	47.2	30.9	16.3	77.4	71	83
Number 4	132	158	9.54	168.7	37.6	18.7	18.9	149.8	58.8	34.2	24.6	123.2	78	84
Number 5	102	115	6.38	97.4	22.4	11.9	10.5	86.9	43.9	26.8	17.1	69.8	77	80
Number 6	95	113	9.17	139.9	31.1	17.6	13.5	126.4	62.6	31.4	31.2	95.2	78	75

VACUUM-DRIED WHITE FISH MEAL DIET

Number 1	94	114	6.96	114.6	19.8	14.3	5.5	109.1	42.8	27.0	15.8	93.3	83	86
Number 2	102	123	7.68	126.5	23.9	17.5	6.4	120.1	46.4	29.8	16.6	103.5	81	86
Number 3	92	111	7.28	119.9	18.2	15.4	2.8	117.1	42.6	29.8	12.8	104.3	85	89
Number 4	101	125	7.98	131.4	20.8	15.5	5.3	126.1	46.0	27.8	18.2	107.9	84	86
Number 5	122	130	5.37	88.4	12.6	9.8	2.8	85.6	44.4	27.5	16.9	68.7	86	80
Number 6	147	158	9.05	149.1	25.1	21.2	3.9	145.2	69.1	41.9	27.2	118.0	83	81

NITROGEN-FREE DIET

Number 1	121	107	6.17	.....	13.8	[a] 2.24	.....	.....	28.1	[b] 24.6	.....	.....	.....	.....
Number 2	127	105	5.84	.....	15.4	[a] 2.64	.....	.....	27.4	[b] 23.6	.....	.....	.....	.....
Number 3	119	110	3.77	.....	9.2	[a] 2.44	.....	.....	23.4	[b] 20.4	.....	.....	.....	.....
Number 4	155	149	6.80	.....	13.7	[a] 2.01	.....	.....	33.1	[b] 21.8	.....	.....	.....	.....
Number 5	125	116	4.09	.....	7.4	[a] 1.81	.....	.....	24.6	[b] 20.4	.....	.....	.....	.....
Number 6	147	134	7.05	.....	18.0	[a] 2.55	.....	.....	36.8	[b] 26.2	.....	.....	.....	.....

[a] Per gram of food.

[b] Per 100 grams live weight.

The data from both rat experiments indicate the probability that the position of the biological value of steam-dried menhaden is intermediate between the biological values of the other two fish meals studied. If the data of the second rat experiment (Table 3) are taken separately, this is more definitely shown. In drawing conclusions regarding the biological values, the author believes that the results of the second rat experiment are much more accurate than those of the first. The potency of the vitamin B concentrate was tested prior to the second experiment, and as a result a much larger quantity was used than in the first experiment. Larger food consumption and more nearly normal growth resulted. Also, as the most erratic biological values in the first experiment occurred with female rats, only males were used in the second experiment. Further, it is likely that during the second experiment the author was more adept in all the technics involved. There were no widely diverging values in the second experiment. This resulted in much smaller probable errors, even though the average values of the second experiment included only half as many observations as were included in the two experiments.

The average biological values obtained in the second rat experiment are 72.7 ± 0.86, 80.2 ± 0.91, and 84.7 ± 0.95 for the proteins of flame-dried menhaden, steam-dried menhaden, and vacuum-dried white meal, respectively. The white fish meal shows an advantage over steam-dried menhaden of 4.5 ± 1.32, the difference being 3.4 times its probable error. The steam-dried menhaden shows an advantage over the flame-dried menhaden of 7.5 ± 1.25, the difference being 6.1 times its probable error. Thus, on the basis of the second rat experiment taken alone, it is not necessary to resort to inference to decide that the position of the steam-dried product is intermediate between the other two fish meals with respect to the utilization of its protein after absorption. The second experiment when taken alone also shows the greater biological value of the white fish meal as compared with the flame-dried menhaden by the more significant difference of 12.0 ± 1.28, the difference being 9.4 times its probable error.

THE SWINE EXPERIMENT

As fish meal is primarily a swine and poultry feed, a further comparison in which only the two commercial meals, flame-dried menhaden and vacuum-dried white meal, were used was made with swine. Two young Berkshire barrows from the same litter, weighing 37.3 and 31.8 kilograms, respectively, were used. Essentially the same technic was followed as with rats. The pigs required a much longer nitrogen-free feeding period before the endogenous level of nitrogen excretion was reached. Daily samples of urine were taken after the second week until what appeared to be a constant and sufficiently low level was reached.

After several attempts, a nitrogen-free ration was mixed with which it was possible to secure more or less uniform excretion of feces. Before collections were made, the composition of the nitrogen-free ration was established at 8 per cent of cellophane, 4 per cent of a mineral mixture, and 88 per cent of starch. The mineral mixture consisted of 40 parts of calcium phosphate, 40 parts of ground limestone, 20 parts of sodium chloride, and 1 part of ferrous sulphate. This ration

was used from five days prior to the first nitrogen-free collection period through this period. It was also used in the final nitrogen-free period. Pig No. 1 was inclined at first to be somewhat irregular in fecal excretion. During the last day of what was intended to be the first 7-day nitrogen-free collection period, he excreted no feces at all. This day was discarded and only six days counted for this first period.

The plan followed in the swine experiment was as follows:

Preliminary period on nitrogen-free ration	19 days.
Collection period on nitrogen-free ration	7 days.
Preliminary period on fish-meal rations	5 days.
Collection period on fish-meal rations	7 days.
Collection period on fish-meal rations	7 days.
Preliminary period on fish-meal rations	5 days.
Collection period on fish-meal rations	7 days.
Collection period on fish-meal rations	7 days.
Preliminary period on nirogen-free ration	5 days.
Collection period on nitrogen-free ration	7 days.

During the first preliminary period on the fish-meal rations, and the two collection periods following, pig No. 1 received the flame-dried menhaden fish meal ration, and pig No. 2 received the vacuum-dried white fish meal ration. During the second preliminary period in which the fish-meal rations were fed, the rations were reversed so that pig No. 1 received the white meal and pig No. 2 the menhaden meal.

As in the rat experiments, in the swine experiment the fish meals were mixed with the nitrogen-free ration at such levels that their proteins formed 10 per cent of the ration. The fish meal replaced part of the starch and minerals of the nitrogen-free ration. Only enough mineral mixture was added to make the total mineral content of each ration 4 per cent. The rations used are shown in Table 4. Cod-liver oil was fed at the rate of 16 g per pig per day.

The pigs were fed twice a day. Throughout the experiment the rations fed each day were entirely consumed; therefore no refused feed had to be accounted for. Each pig vomited slightly once during the last fish-meal collection period, but the amount involved less than 1 gram of dry matter. It was concluded that the amount of nitrogen lost was insignificant as compared with the weekly nitrogen intake.

TABLE 4.—*Composition of the fish meal rations used in the swine feeding experiment*

Constituent	Composition of the flame-dried menhaden fish meal ration	Composition of the vacuum-dried white fish meal ration
	Parts	*Parts*
Fish meal	15.88	14.83
Starch	73.62	74.17
Mineral mixture	.50	1.00
Cellophane	8.00	8.00
Yeast	2.00	2.00

TABLE 5.—*Data obtained with swine in the experiment to determine the protein efficiency of fish meals*

Pig	Initial weight	Final weight	Average daily food intake	Average daily N intake	Average daily fecal N	Estimated daily metabolic N	Average daily food N in the feces	Average daily absorbed N	Average daily urinary N	Estimated daily endogenous N	Average daily food N in urine	Average daily food N utilized	Digestion coefficient	Biological
	Kgm.	*Kgm.*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Per cent*	*Per cent*
NITROGEN-FREE RATION														
No. 1 [a]	37.3	36.9	700	.....	0.68	[b] 0.97	.....	.....	1.98	[c] 0.053	.....	.....	.....	.....
No. 2	31.8	31.3	600	.....	.54	[b] .90	.....	.....	2.12	[c] .067	.....	.....	.....	.....
FLAME-DRIED MENHADEN FISH MEAL RATION														
No. 1	38.3	39.6	750	11.99	3.18	.69	2.49	9.50	4.94	1.95	2.99	6.51	73	69
No. 1	39.6	40.2	750	11.99	3.36	.68	2.68	9.31	5.13	1.92	3.21	6.10	72	66
No. 2	35.6	36.9	750	11.99	3.06	.83	2.23	9.76	4.60	2.11	2.49	7.27	74	71
No. 2	36.9	37.7	750	11.99	2.98	.86	2.12	9.87	4.91	2.09	2.82	7.05	75	74
VACUUM-DRIED WHITE FISH MEAL RATION														
No. 1	42.3	44.3	850	13.83	1.72	.72	1.00	12.83	4.84	1.94	2.90	9.93	83	77
No. 1	44.3	45.7	850	13.83	1.34	.71	.63	13.20	4.76	1.93	2.83	10.37	90	79
No. 2	32.7	33.7	650	10.58	1.03	.64	.39	10.19	3.92	2.12	1.80	8.38	90	82
No. 2	33.7	35.1	650	10.58	1.04	.66	.38	10.20	3.74	2.12	1.62	8.58	90	84
NITROGEN-FREE RATION														
No. 1	45.4	45.6	775	.....	.61	[b] .78	.....	.....	1.81	[c] .040	.....	.....	.....	.....
No. 2	37.4	37.6	675	.....	.83	[b] 1.22	.....	.....	1.96	[c] .052	.....	.....	.....	.....

[a] This is a 6-day collection period instead of a 7-day period.

[b] Fecal nitrogen per kilogram of dry feed. These values were used in estimating the metabolic nitrogen in the feces in the fish-meal periods. The change in the ratio of metabolic nitrogen to dry matter consumed from the first to last periods was assumed to occur in a linear fashion.

[c] Urinary nitrogen per kilogram of body weight. These values were used in estimating the endogenous (body) nitrogen in the urine in the fish-meal periods, the same assumption of a linear variation from the first to last periods being made as in the case of the metabolic nitrogen in the feces.

The results of the swine experiment are shown in Table 5. This limited experiment, in which only two pigs were used, is in reality of greater significance than would be the case if the data were not in agreement with those from the 12 rats. The results with even two pigs are significant under such circumstances. Attention is called to the fact that the rat rations and the pig rations were not entirely comparable. The fish-meal proteins were present in each at a 10 per cent level by analysis. However, because of the high fat content of the rat rations the percentage of protein calories was only 8 per cent, whereas with the swine rations the cellophane and mineral content caused the percentage of protein calories to be 12 per cent, if it is assumed that no calories are derived from the cellophane.

An inspection of the digestion coefficients and biological values in Table 5 shows clearly that the pig experiment supports the work with rats in showing the superiority of the proteins of the white fish meal. Since the data were furnished by only two animals, the values were not averaged nor treated statistically. It is evident however, that the protein of the vacuum-dried white fish meal was decidedly more efficient than the protein of the flame-dried menhaden fish meal, with each pig, in both digestion and anabolism. It will be noticed that throughout the experiment pig No. 2 tended to utilize protein more efficiently than pig No. 1.

The experiments described in this paper confirm those of Maynard, Bender, and McCay [6] in showing that the products studied rank in the following order as regards protein efficiency: Vacuum-dried white fish meal, steam-dried menhaden meal, flame-dried menhaden meal. In addition, they supply more specific and quantitative information in showing that the differences result in part from differences in digestibility and in part from differences in the utilization of the absorbed nitrogen. Further, they indicate that the results obtained with the rats hold also for growing pigs and thus furnish further evidence as to the usefulness of the rat for pilot experiments with swine rations. In view of the fact that fish meals are used primarily as protein supplements in both swine and poultry rations, the results obtained with the two commercial meals are of obvious practical importance. They suggest the desirability of further studies, not only of various products now sold for stock feeding, but also of the conditions both as to raw material and processing which provide proteins of high biological value.

SUMMARY

In two nitrogen-balance studies involving a total of 12 growing rats the products studied were found to rank in the following order as regards the digestibility of their proteins: Vacuum-dried white fish meal, steam-dried menhaden meal, flame-dried menhaden meal. A comparison of the white meal and the flame-dried menhaden by the same procedure with two growing pigs produced results similar to those obtained with the rats.

In both rat experiments and in the swine experiment the vacuum-dried white meal proved significantly superior to the flame-dried

[6] MAYNARD, L. A., BENDER, R. C., and MCCAY, C. M. Op. cit.

menhaden as regards the utilization of the absorbed nitrogen. In both rat experiments the white meal proved numerically superior in this respect to the steam-dried menhaden, and the latter numerically superior to the flame-dried product, but these differences were biometrically significant in the case of the second experiment only.

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